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Gastrointestinal Parasites at the Human–Wildlife Interface:

Diversity, Transmission and Consequences for Host Health

Habilitation Thesis

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Brno 2026

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Abstract

This habilitation thesis is presented as a selection of 26 scientific publications accompanied by an introductory commentary. Although the studies address different parasite groups, host species and ecological settings, they are connected by a common focus on gastrointestinal parasites, their diversity, host specificity, transmission ecology and consequences for host health. Particular attention is given to non-human primates and parasites occurring at the human–wildlife interface. The thesis is organised into four main thematic areas. The first focuses on parasite diversity and the application of molecular approaches, particularly metabarcoding, to identify parasite species and genetic lineages that are difficult to distinguish using conventional methods. The second addresses host specificity, parasite sharing and zoonotic potential, with particular emphasis on *Strongyloides* and strongylid nematodes circulating among humans, non-human primates and other mammals. The third examines how ecological and anthropogenic factors, including habitat disturbance and human activities, influence parasite communities and infection patterns. The final part focuses on the health consequences of gastrointestinal parasites in wild primates, particularly the emerging strongylid-associated disease problems in mountain gorillas. The broader conclusion of this habilitation is that gastrointestinal parasites need to be studied as part of dynamic systems involving multiple hosts and their shared environments. This is particularly important at the human–wildlife interface, where parasite circulation may have implications for human health, domestic animals and wildlife conservation. A One Health approach provides a useful framework for bringing these components together, but its success depends on understanding the biology of the parasites themselves. By combining increasingly detailed molecular identification with ecological and health-related information, we can move beyond describing parasite diversity towards a better understanding of the processes that determine parasite transmission and its consequences.

Abstrakt

Tato habilitační práce je předkládána jako soubor 26 vybraných vědeckých publikací doplněný úvodním komentářem. Přestože se jednotlivé studie zabývají různými skupinami parazitů, hostitelskými druhy a ekologickými podmínkami, spojuje je společné zaměření na gastrointestinální parazity, jejich diverzitu, hostitelskou specificitu, způsob přenosu a důsledky infekcí pro zdraví hostitelů. Zvláštní pozornost je věnována nehumánním primátům a parazitům vyskytujícím se na rozhraní mezi člověkem a volně žijícími živočichy. Práce je rozdělena do čtyř hlavních tematických oblastí. První se zaměřuje na diverzitu parazitů a využití molekulárních metod, zejména metabarcodingu, k identifikaci druhů a genetických linií parazitů, které je obtížné rozlišit konvenčními metodami. Druhá oblast se zabývá hostitelskou specificitou, sdílením parazitů mezi hostiteli a zoonotickým potenciálem, se zvláštním důrazem na hlístice rodu *Strongyloides* a strongylidní hlístice cirkulující mezi lidmi, nehumánními primáty a dalšími savci. Třetí oblast zkoumá, jak ekologické a antropogenní faktory, včetně narušování přirozených stanovišť a lidských aktivit, ovlivňují společenstva parazitů a výskyt infekcí. Poslední část

se zaměřuje na zdravotní důsledky gastrointestinálních parazitárních infekcí u volně žijících primátů, zejména na nově se objevující zdravotní problémy spojené se strongylidními infekcemi u goril horských. Obecným závěrem této habilitační práce je, že gastrointestinální parazity je nezbytné studovat jako součást dynamických systémů zahrnujících více hostitelských druhů a jimi sdílené prostředí. To je zvláště důležité na rozhraní mezi člověkem a volně žijícími živočichy, kde může mít cirkulace parazitů důsledky pro lidské zdraví, domácí zvířata i ochranu volně žijících živočichů. Přístup One Health poskytuje vhodný rámec pro propojení těchto složek, jeho úspěšné uplatnění však závisí na porozumění biologie samotných parazitů. Propojením stále podrobnější molekulární identifikace s ekologickými informacemi a poznatky o zdravotních důsledcích infekcí se můžeme posunout od pouhého popisu diverzity parazitů k hlubšímu pochopení procesů, které určují jejich přenos a důsledky.

Acknowledgement

I would like to thank all the people who have helped me throughout my scientific journey – my colleagues, friends and family.

I have been very lucky because, throughout my scientific career, I have always felt tremendously supported. At the beginning, this support came mainly from my former supervisors, Klára Petrželková and David Modrý. They taught me something very important: a passion for science, and the courage to follow questions that I find interesting. It does not really matter whether others see you as the right person to do particular research, or whether they consider the topic important enough. If you believe something is worth exploring, you should give it a try. Over almost 20 years of working more or less together, Klára and David have always supported me, encouraged me and offered valuable advice, for which I am deeply grateful. As I grew scientifically and gradually became more independent, I was fortunate to receive support from many other scientists, both in the Czech Republic and abroad. I will avoid naming them individually, simply because there are so many that I would be afraid of leaving someone out. Their encouragement, trust and willingness to share their experience have meant a great deal to me. They helped me believe that I had chosen the right scientific path and that I should continue following it. Such support is especially important during those moments when you begin to question whether you should stay in science at all.

After completing my PhD, I worked towards establishing my own research group. This was another important milestone, particularly because I began supervising PhD students. I would especially like to thank Bethan Mason, Vladislav Ilík, Eva Nosková and Kelly Sambucci, whom I have had the pleasure of supervising or co-supervising. Working with you throughout your PhDs has been an amazing experience. This habilitation thesis is, to a large extent, built on the research we have done together, and I am very grateful for everything I have learned from you along the way. I would also like to thank all my undergraduate and Master's students. Working with students has always been one of the most inspiring parts of my scientific life.

My thanks also go to all the members of my previous and current research teams. We have shared so many experiences, from fieldwork and long days in the laboratory to seemingly never-ending scientific discussions. Much of the research presented in this thesis relies on samples collected in different parts of the world, and I am particularly grateful to all the local scientists, students, field assistants and collaborators who have contributed to sample and data collection. Without their help, much of this work would simply not have been possible. To my current team, thank you for your ideas, enthusiasm and for constantly inspiring and motivating me to do better science.

I am equally lucky in my personal life, where I am surrounded by people who have always supported me. I am especially grateful to those who interrupt me while I am working on my habilitation, because life is certainly not only about work. To my three beloved children, who remind me every day what really matters in life. To my parents, my sister and the rest of my family, who help make all of this possible. And to my husband – simply for everything.

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1. Introduction: Gastrointestinal parasites at the human–wildlife interface

Parasites are integral components of natural ecosystems and form long-standing associations with their hosts (Hatcher and Dunn, 2011; Buckingham and Ashby, 2022). Gastrointestinal parasites and other eukaryotic symbionts are particularly diverse, ranging from organisms that persist with little apparent effect on host health to important pathogens capable of causing severe disease (Maizels and Yazdanbakhsh, 2003; Allen and Maizels, 2011). Individual hosts frequently harbour multiple parasite taxa simultaneously, forming complex communities whose composition reflects the interplay between parasite biology, host evolutionary history, ecology and environmental exposure (Hatcher and Dunn, 2011; Rohr et al., 2020). Understanding these communities therefore requires moving beyond the simple detection of infection towards determining which parasites are present, which hosts they infect, what determines their distribution, and under which circumstances infection has consequences for host health.

These questions become particularly important at the human–wildlife interface. Human activities increasingly modify natural habitats and the ways in which humans, wildlife and domestic animals share landscapes and resources (Karesh et al., 2012; Rulli et al., 2025). For many gastrointestinal parasites, transmission does not require direct contact between hosts: infective stages released into the environment may persist in soil, vegetation, food or water, or transmission may involve a range of intermediate hosts or vectors. The human–wildlife interface is therefore better understood as an ecological system connecting multiple hosts through shared environments rather than simply as a zone of direct contact between humans and wild animals (Wells et al., 2018). Within such systems, transmission can potentially occur in multiple directions, making simple distinctions between reservoir and recipient hosts difficult.

Non-human primates provide a particularly informative model for investigating these processes. Their close evolutionary relationship with humans may facilitate the establishment of some parasites following cross-species exposure, while differences in ecology, behaviour, habitat use and degree of contact with humans provide opportunities to investigate how ecological factors modify parasite sharing (Davies and Pedersen, 2008; Cooper et al., 2012). At the same time, primates themselves represent important conservation targets, meaning that parasite transmission across the human–wildlife interface is relevant not only to human health but also to the health of endangered wildlife (Gillespie et al., 2008).

Assessing parasite host specificity (expressed as the range of hosts in which a parasite can establish and maintain an infection) is a key element of parasite transmission ecology and is usually considered one of the basic biological traits of parasite taxa, particularly at the species level (Wells and Clark, 2019). However, parasite species boundaries are often difficult to define, and morphologically similar developmental stages may conceal considerable taxonomic and genetic diversity (Nadler and Pérez-Ponce de León, 2011). Host association does not necessarily resolve this problem: parasites found in different hosts may represent separate host-specific taxa, genetically differentiated populations or occasional spillover infections, rather than a single generalist parasite circulating among several host species. Conversely, individual hosts may harbour several closely related and morphologically

indistinguishable taxa simultaneously. Species delimitation is therefore not merely a taxonomic issue; it directly influences how host range, transmission, reservoir status and zoonotic potential are interpreted (de Queiroz, 2007; Nadler and Pérez-Ponce de León, 2011). These problems are especially pronounced in wildlife, where studies frequently depend on non-invasive samples and morphologically informative parasite stages are rarely available. Molecular studies across parasite groups have consequently revealed extensive diversity that remained hidden under morphology-based classifications.

Advances in molecular methods have fundamentally changed the resolution at which this diversity can be investigated. Targeted molecular markers, high-throughput amplicon sequencing and increasingly genome-wide approaches make it possible to distinguish parasite species and genetic lineages, resolve mixed infections and characterize complex parasite communities (Criscione et al., 2005; International Helminth Genomes Consortium, 2019). However, greater molecular resolution does not automatically provide biological understanding. Molecular diversity must be interpreted in relation to parasite morphology, host association, ecology and transmission biology (Nadler and Pérez-Ponce de León, 2011; Rojas et al., 2025), while incomplete reference databases and marker-dependent patterns continue to constrain taxonomic interpretation (Valkiūnas et al., 2008). An integrative approach is therefore essential to translate genetic variability into biologically meaningful parasite diversity. This is particularly important because species and lineage identification provides the foundation for all subsequent questions concerning host specificity and cross-species transmission.

Even when parasite identity is sufficiently resolved, occurrence across host species reflects an interaction between host compatibility and ecological opportunities for transmission (the compatibility and encounter filters; Combes, 2001). The ecological context of these relationships is itself dynamic. Environmental conditions, host population structure, behaviour and anthropogenic disturbance can alter opportunities for parasite transmission and thereby restructure communities of gastrointestinal symbionts (Wells and Clark, 2019; Rulli et al., 2025). Importantly, these effects need not be uniform. Different parasites respond differently to environmental change, and the same disturbance may have contrasting effects in different host species. Parasite communities should therefore not be viewed as static characteristics of their hosts, but as dynamic outcomes of interactions among hosts, parasites and their environments.

Parasites are commonly considered pathogenic agents, as parasitism is generally associated with a potential negative impact on host fitness. However, parasite presence cannot be equated directly with disease (Sánchez et al., 2018). Many gastrointestinal parasites form persistent associations with their hosts without obvious clinical consequences, whereas others may cause severe pathology only under particular circumstances (Allen and Maizels, 2011). Even within the same host population, apparently similar infections can have very different outcomes. Understanding parasite-associated disease therefore requires asking not only whether a parasite is present, but when and why an established infection becomes pathogenic. This distinction is particularly important in wildlife, where information on clinical

outcomes is often limited and where changes in parasite communities may occur simultaneously with changes in host populations and their environments (Sánchez et al., 2018; Chrétien et al., 2023).

Together, these considerations place gastrointestinal parasites within a system extending from parasite genetic diversity through host associations and ecological transmission to consequences for host health at individual or population levels. A One Health perspective arises naturally from this complexity: humans, wildlife, domestic animals and their shared environments cannot be treated as independent components of parasite transmission systems (Karesh et al., 2012).

The research synthesized in my habilitation thesis addresses three cardinal and interconnected questions. First, how can the hidden taxonomic and genetic diversity of gastrointestinal parasites be revealed and reliably characterized? Second, what determines host specificity, parasite sharing and the structure of parasite communities across hosts and environments? Third, under which conditions does infection remain an apparently tolerated component of the gastrointestinal ecosystem, and when does it contribute to disease? The following chapters address these questions by progressively connecting molecular resolution with host ecology and evolutionary relationships, environmental and anthropogenic change, and ultimately the consequences of infection for host health.

2. Parasite diversity: from microscopy to metabarcoding and genomics

Reliable identification of parasites is fundamental to understanding their diversity, ecology, transmission and effects on host health. Yet, for many gastrointestinal helminths, and particularly for strongylid nematodes, limited taxonomic resolution has historically represented a major obstacle to understanding their ecological and health impacts (Nadler and Pérez-Ponce de León, 2011). Strongylids commonly occur as complex multispecies infections in mammalian hosts, while their diagnostic stages in faeces (eggs or larvae) are often morphologically indistinguishable (Avramenko et al., 2015; Pafčo et al., 2018). This problem is particularly pronounced in studies of wildlife, where non-invasive faecal sampling is frequently the only feasible approach and adult worms, on which classical species descriptions are largely based, are rarely available. Consequently, conventional coproscopic surveys have often reported infections collectively as “strongylids”, concealing considerable taxonomic and genetic diversity and limiting conclusions about parasite ecology, host specificity and transmission.

The development of molecular approaches has progressively overcome some of these limitations and has been a central methodological component of my research. Importantly, this development should not be regarded as a simple replacement of traditional parasitology by increasingly sophisticated molecular techniques. Different approaches provide different types of biological information. Microscopy remains essential for detecting parasite stages, estimating infection intensity and linking molecular sequences to morphologically identified organisms. Appropriately selected molecular markers allow species or lineage identification and phylogenetic comparison (Gasser, 2025). High-throughput amplicon sequencing extends this resolution to the characterisation of complex mixed infections and entire parasite communities, whereas genome-wide approaches provide access to

population structure, evolutionary history and potentially the genetic basis of host adaptation and other phenotypic traits (International Helminth Genomes Consortium, 2019). The value of increasing molecular resolution therefore lies not simply in detecting more diversity, but in enabling biological questions that could not previously be addressed.

2.1 From morphological identification to molecular resolution

Traditional diagnosis of gastrointestinal nematodes is largely based on the detection of eggs or larvae in faeces. Although cultivation and morphological examination of third-stage larvae can improve identification (usually to the genus level), reliable species identification remains difficult for the majority of strongylid taxa. Adult worms provide considerably greater morphological resolution, but obtaining them generally requires necropsy or, in human and veterinary settings, recovery from faeces following anthelmintic treatment. Thus, the availability of adult worms is particularly limited in studies of endangered wildlife. Moreover, many species were described decades ago from relatively few specimens, and historical type material may be unavailable or preserved in a manner incompatible with subsequent molecular characterization. The result is a persistent gap between classical taxonomy and sequence-based identification. Molecular approaches usually depend on reference sequences, but reliable reference sequences ultimately depend on parasites that have themselves been accurately identified (Nadler and Pérez-Ponce de León, 2011).

Targeted molecular approaches have substantially increased the resolution achievable from non-invasively collected samples. Ribosomal markers, particularly ITS-1 and ITS-2 and variable regions of 18S rRNA, together with mitochondrial markers such as cox1, have been used for species identification, phylogenetic reconstruction and investigation of within-species genetic diversity (Gasser, 2025). Similar methodological obstacles also complicate our understanding of other nematode groups, including *Strongyloides* (Rhabditida), whose diversity is difficult to resolve using conventional diagnostic approaches. Individual hosts may harbour more than one *Strongyloides* species or genetically distinct lineages, while their diagnostic stages provide limited morphological information for distinguishing such mixed infections (Bradbury et al., 2021).

In our review of *Strongyloides* genotyping approaches (Bradbury et al., 2021), we evaluated the range of molecular markers available not only for resolving parasite diversity but also for addressing questions related to transmission and zoonotic potential. Four hypervariable regions within 18S rRNA have been used as potential genotyping targets, of which HVR-I and HVR-IV have become the most widely applied. HVR-IV provides useful discriminatory information among species and major lineages, whereas mitochondrial cox1 generally provides greater resolution of intraspecific variation. Combining these markers has therefore made it possible to distinguish closely related *Strongyloides* lineages and investigate their geographical distribution and occurrence across different host species. This increased molecular resolution has substantially changed our understanding of *Strongyloides* diversity and parasite–host associations. Genotyping studies have revealed considerable genetic structure within both

S. stercoralis and *S. fuelleborni*. In *S. stercoralis*, distinct genetic lineages differ in their host associations, whereas *S. fuelleborni* shows pronounced geographical structuring, with related genotypes occurring in sympatric primate species.

2.2 Metabarcoding: from individual parasites to parasite communities

Deep amplicon sequencing (DAS) encompasses applications ranging from metabarcoding of mixed-species communities to targeted sequencing of genetic variants within parasite populations. Its principal advantage in studies of complex parasite communities lies in the simultaneous recovery of sequence information from multiple taxa present within the same biological sample. For strongylids, metabarcoding of the ITS-2 region has proved particularly useful because this marker combines sufficient taxonomic resolution for many taxa with amplification of a broad range of nematodes from mixed DNA templates (Pafčo et al., 2018). Rather than classifying a sample as simply positive for "strongylids", it becomes possible to reconstruct communities comprising multiple amplicon sequence variants (ASVs), assign many of them taxonomically, and compare community composition among hosts, populations or environments.

However, greater sequencing depth does not automatically result in more reliable biological information. As emphasized in our synthesis of ten principles for implementing DAS in parasitology (Šlapeta et al., 2026), the method should be selected according to the biological question and used as a complement to, rather than a replacement for, conventional parasitological and molecular approaches (Figure 1). The observed sequence composition is influenced by the number of parasites contributing DNA, extraction methods, marker choice, primer specificity, amplification efficiency, and sequencing and bioinformatic procedures. Low parasite abundance is especially problematic when sequence proportions are interpreted quantitatively, because DNA extracted from a small faecal subsample may originate from only a few individual eggs or larvae. PCR amplification can further distort the original community if templates differ in concentration or primer-binding efficiency. Technical replication and rigorous filtering can reduce some of these sources of error but cannot eliminate biological sampling limitations (Šlapeta et al., 2026).

Marker selection is similarly critical. No universal marker provides optimal resolution across all parasite groups or for all biological questions. Highly conserved loci may amplify a broad range of targets but provide insufficient taxonomic resolution, whereas more variable markers can distinguish closely related taxa but may introduce amplification biases or complicate comparisons with incomplete reference databases. This is particularly relevant when ASVs are interpreted as species or when sequence diversity is used to infer cryptic taxa. The limitations of reference databases remain one of the most important constraints on parasite metabarcoding: sequence-based identification cannot be more reliable than the taxonomic identification of the material from which reference sequences originated. Deep sequencing therefore increases rather than eliminates the need for integrative taxonomy. When supported by well-curated reference databases, however, DAS permits identification independent of

parasite developmental stage and makes high-resolution analysis possible even in systems where material for morphology-based identification cannot routinely be obtained (Šlapeta et al., 2026).



Figure 1. Ten simple rules for implementing deep amplicon sequencing in parasitology. Successful application of deep amplicon sequencing begins with a clearly defined research question. The central question should guide whether this approach enhances and is capable of addressing the biological or diagnostic challenge at hand. Surrounding this core, the rules aim to balance resolution, feasibility and reproducibility. Šlapeta et al., 2026.

These methodological principles have underpinned our application of metabarcoding across substantially different host systems. In western lowland gorillas (*Gorilla gorilla gorilla*), we used HTS to characterize strongylid communities from faecal samples collected at five localities across the Congo Basin (Mason et al., 2022a). The analysis detected strongylid ASVs representing eight genera, demonstrating a level of diversity that cannot be recovered from routine coproscopy. Importantly, this study combined strongylid metabarcoding with characterization of the bacterial microbiome, thereby moving beyond a simple inventory of parasite taxa towards an analysis of the gastrointestinal ecosystem. Variation occurred among localities in both bacterial and strongylid communities, and several strongylid ASVs, particularly those belonging to *Necator* and *Oesophagostomum*, showed patterns of covariation with bacterial taxa.

The same methodological framework also allowed comparisons across much broader host and geographical contexts. Applying ITS-2 metabarcoding to non-human primates from South-East and East

Asia revealed a markedly different pattern from that observed in African primates (Mason et al., 2024). Strongylid communities were comparatively simple and strongly dominated by *Oesophagostomum aculeatum*. At the same time, considerable sequence variation was observed within *Oesophagostomum* (Figure 2). Interpretation of this variation illustrates an important limitation of single-marker metabarcoding: although ITS-2 performs well for distinguishing many known species, its substantial intraspecific variability means that divergent ASV clusters cannot automatically be interpreted as cryptic species. Resolving their taxonomic status therefore requires additional markers, particularly mitochondrial loci such as cox1, ideally combined with morphological examination of adult worms.

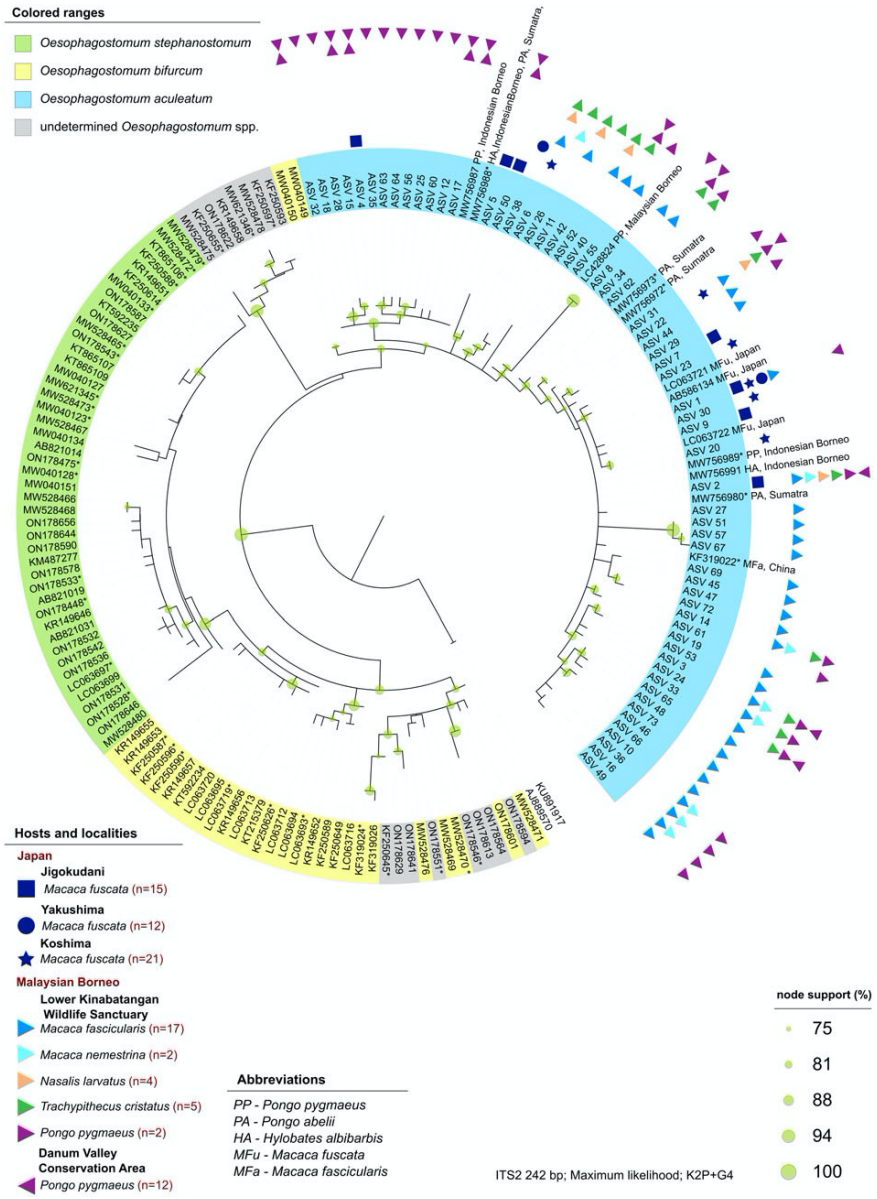


Figure 2. Maximum likelihood cladogram of *Oesophagostomum* ITS-2 region (245 bp), computed in IQ tree3 by model K2P + G4, using *O. dentatum* as an outgroup. The tree topology was tested by 1000 replicates of ultrafast bootstrap4 and SH-like aLRT5. Green circles mark nodes with support higher than 75%, with circle size depicting value. Sequences from GenBank are labelled by accession number. Mason et al., 2024.

2.3 Extending metabarcoding beyond wildlife: surveillance and treatment response

The utility of metabarcoding is not restricted to wildlife systems. We applied the same general approach to investigate whether HTS could provide information relevant to parasite surveillance and evaluation of anthelmintic treatment in beef cattle (Pafčo et al., 2024). ITS-2 metabarcoding across Czech cattle herds revealed communities dominated by *Ostertagia ostertagi* and *Oesophagostomum* spp., with substantial representation of *Trichostrongylus* and *Cooperia*, while representatives of other genera, such as *Bunostomum*, *Nematodirus* and *Chabertia*, occurred less frequently. Comparative material from cattle in Dobrogea, Romania, revealed a substantially different community dominated by *Haemonchus placei*, illustrating the ability of metabarcoding to identify regional variation that is obscured when infections are assessed only through strongylid egg counts.

More importantly, the study demonstrated how community-level sequencing can address applied questions. Following ivermectin treatment on two Czech farms, strongylid community composition shifted, with some taxa decreasing in relative abundance and others no longer detected, while *Cooperia* and *Ostertagia* became relatively dominant in some samples (Figure 3). Such changes are particularly relevant in the context of anthelmintic resistance, as deep sequencing can reveal taxon-specific shifts in community composition that may be obscured by aggregate faecal egg counts.

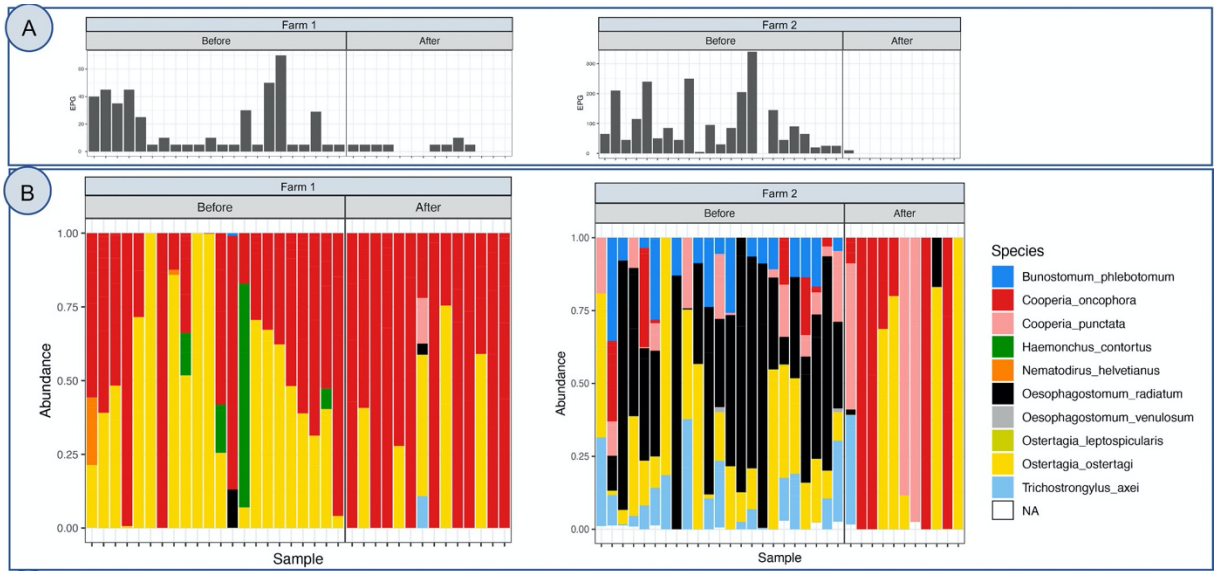


Figure 3. Bar-plot shows A) faecal egg counts and B) community composition of strongylid nematodes at species level (each column represents the relative abundance of ITS-2 amplicon sequence variants per sample) of strongylid nematodes in beef cattle in the Czech Republic before and after anthelmintic treatment. Pafčo et al., 2024.

2.4 *Strongyloides*: when molecular resolution changes epidemiological interpretation

The methodological challenges outlined above for *Strongyloides* have direct consequences for epidemiological interpretation. This is especially relevant for *S. fuelleborni*, a parasite primarily associated with non-human primates but also known to infect humans. The taxonomic status and epidemiology of *S. fuelleborni* in New Guinea have remained particularly uncertain. Human infections there were historically attributed to *S. fuelleborni kellyi* (Viney et al., 1991; Ashford et al., 1992), but the identity of this taxon has remained unresolved (Zhao et al., 2025).

In our recent study of longitudinal faecal samples from infants in Papua New Guinea, we combined *Strongyloides*-specific qPCR with metabarcoding and sequencing of several informative loci, including 18S rRNA hypervariable regions and mitochondrial cox1 (Zhao et al., 2025). Molecular evidence of *Strongyloides* infection was found in eight of 19 infants. Seven were infected with *S. fuelleborni*, belonging to the Asia-Pacific lineage, whereas one harboured a genetically distinct *Strongyloides* lineage that may represent an undescribed species. These findings raise questions about the taxonomic identity of the historically described *S. fuelleborni kellyi*, which may correspond to the Asia-Pacific lineage of *S. fuelleborni*, rather than to the genetically distinct taxon detected in our study. The data therefore indicate that at least two *Strongyloides* taxa circulate in this population. Historically, *S. fuelleborni kellyi* was also implicated in swollen belly syndrome, a severe and sometimes fatal disease of infants. Our findings suggest that historical epidemiological data based largely on microscopy may have conflated infections caused by different *Strongyloides* taxa. This leaves important questions unresolved, including which parasite was responsible for swollen belly syndrome and how *S. fuelleborni* is maintained in Papua New Guinea in the absence of non-human primates, its usual reservoir hosts.

The value of increased resolution in *Strongyloides* diversity extends beyond epidemiological surveillance and is also important for investigating potential differences in pathogenicity among parasite lineages. *Strongyloides stercoralis* circulating in dogs comprises two major lineages: the potentially zoonotic lineage A, which occurs in humans and dogs, and the predominantly dog-associated lineage B (Bradbury and Streit, 2024). In a fatal case of disseminated strongyloidiasis in a dog, in which large numbers of *S. stercoralis* were associated with severe intestinal pathology, we used HVR-IV high-throughput sequencing to characterize the infection (Nosková et al., 2024a). More than 97,000 high-quality reads were assigned exclusively to the potentially zoonotic lineage A, with no evidence of the canine-restricted lineage B. Additional molecular markers also supported assignment to lineage A. Although a single case cannot establish lineage-specific pathogenicity, lineage A has also been identified in other molecularly characterized fatal canine infections, whereas the pathogenic potential of lineage B remains unknown.

2.5 Beyond amplicons: towards parasite genomics

Despite the substantial increase in resolution provided by metabarcoding, amplicon-based approaches interrogate only a small fraction of the parasite genome. They are highly effective for identifying taxa, describing community composition and tracking selected genetic variants, but are inherently limited for answering questions involving genome-wide population structure, demographic history, selection or the genetic basis of adaptation. For these questions, whole-genome sequencing offers substantially greater resolution (International Helminth Genomes Consortium, 2019).

Hookworms illustrate both the potential and the current limitations of moving from targeted molecular markers to genome-wide analyses. These nematodes are among the most important soil-transmitted helminths of humans, but also include a much broader diversity of species infecting domestic

and wild mammals. As discussed in our review of hookworm genomics (Ilík et al., 2024), among more than 100 described hookworm species, genome sequences were available for only four species at the time of publication: *Necator americanus*, *Ancylostoma duodenale*, *A. ceylanicum* and *A. caninum*. Moreover, these genomes were generated from laboratory-maintained parasites, which may not fully represent the genetic diversity of natural populations. Although molecular studies have revealed considerable genetic diversity among hookworms, their genome-wide population structure, evolutionary history and the genetic basis of potential host adaptation remain largely unexplored. Genome-wide analyses of parasites collected from naturally infected hosts could help address these questions.

As illustrated by the studies discussed in this chapter, these approaches provide complementary levels of information. Microscopy allows the assessment of parasite occurrence, morphology and infection intensity; targeted molecular markers enable species identification and lineage assignment; and metabarcoding resolves complex parasite communities and mixed infections. Genome-wide analyses extend these possibilities to questions of population connectivity, evolutionary history and adaptation that cannot be adequately addressed using individual genetic markers.

3. Host specificity, parasite circulation among host species and zoonotic potential

Parasite host specificity is generally defined as the degree to which a parasite is restricted to a particular host species or range of host species in which it can establish and reproduce (Poulin and Keeney, 2008). Parasites differ considerably in their host specificity. Some are restricted to a single host species or a narrow group of closely related hosts, whereas others infect phylogenetically diverse species (Cooper et al., 2012). Understanding the processes that determine these patterns is fundamental to parasite ecology and evolution, but also to assessing the risk of zoonotic transmission. This is particularly relevant at the human–wildlife interface, where anthropogenic pressure, habitat modification and changes in land use can increase spatial overlap among humans, wildlife and domestic animals (Plowright et al., 2017; Faust et al., 2018). Non-human primates represent an especially important system in this context because their close evolutionary relationship with humans may facilitate the establishment of parasites and other pathogens following cross-species exposure (Pedersen et al., 2005; Cooper et al., 2012). At the same time, transmission can occur in both directions: non-human primates may act as sources of human infections, while pathogens introduced by humans can represent important threats to endangered primate populations.

However, the occurrence of genetically related or even apparently identical parasites in different host species should not automatically be interpreted as evidence of zoonotic transmission. Parasite sharing requires at least two conditions. First, hosts must encounter the same infective stages; second, the parasite must be capable of establishing itself in the new host. In the framework proposed by Combes (2001), these processes are described as the encounter filter and the compatibility filter. The encounter filter is predominantly ecological and depends on spatial and temporal overlap between hosts and infective parasite stages, whereas the compatibility filter reflects the physiological and immunological

conditions determining whether a parasite can survive and reproduce after exposure. For closely related hosts living in the same environment, both barriers may be relatively weak, increasing the probability of parasite sharing. Nevertheless, their relative importance differs among parasite taxa and transmission modes (Cooper et al., 2012). This distinction is particularly important for gastrointestinal parasites because many are transmitted indirectly through a shared environment rather than through physical contact between hosts. Parasite stages shed in faeces, including eggs and larvae, can contaminate soil, vegetation, food or water and subsequently infect another host. Thus, spatial overlap and the use of shared environments may be epidemiologically more important than direct contact.

Molecular approaches have substantially improved our ability to investigate these processes. As discussed in the previous chapter, morphologically similar parasite stages can conceal considerable species and lineage diversity. A parasite apparently occurring in several host species on the basis of microscopy may represent several host-specific taxa, while genetically identical lineages may circulate among hosts previously assumed to harbour different parasites (Poulin and Keeney, 2008; Nadler and Pérez-Ponce de León, 2011). Nevertheless, even the detection of identical haplotypes in different hosts does not establish the direction of transmission, the frequency of cross-species infections or whether a parasite can maintain transmission within a new host population. These limitations are important when assessing zoonotic potential, as illustrated by studies of amoebae of the genus *Entamoeba*, where molecular identification is essential for distinguishing lineages with different host associations and pathogenic potential.

3.1 Host phylogeny versus ecological overlap

Much of our research on sympatric primates in Central Africa has focused on parasite circulation among host species sharing the same habitat. In the Dzanga-Sangha Protected Areas (DSPA) in the Central African Republic, western lowland gorillas (*Gorilla gorilla gorilla*), central chimpanzees (*Pan troglodytes troglodytes*), agile mangabeys (*Cercocebus agilis*) and humans share the same forest ecosystem but differ in their phylogenetic relationships, habitat use and degree of spatial overlap. This provides a natural system for investigating whether strongylid community composition primarily reflects host evolutionary relationships or ecological opportunities for transmission in a shared habitat.

Using ITS-2 metabarcoding, we identified 85 strongylid haplotypes representing at least nine genera. Both host-specific and shared haplotypes were detected, with *Oesophagostomum* and *Necator* representing the dominant genera (Pafčo et al., 2019). *Necator americanus*, generally considered the principal human hookworm, was strongly associated with humans, whereas *N. gorillae* was widespread among gorillas and chimpanzees but was also detected in humans (Figure 4). Importantly, strongylid communities of BaAka trackers, who spent substantial time in the forest, were more similar to those of gorillas than were communities of their relatives living predominantly in villages. Conversely,

increasing gorilla habituation, and therefore closer contact with humans, was not associated with a greater occurrence of human-associated strongylids in gorillas (Pafčo et al., 2019).

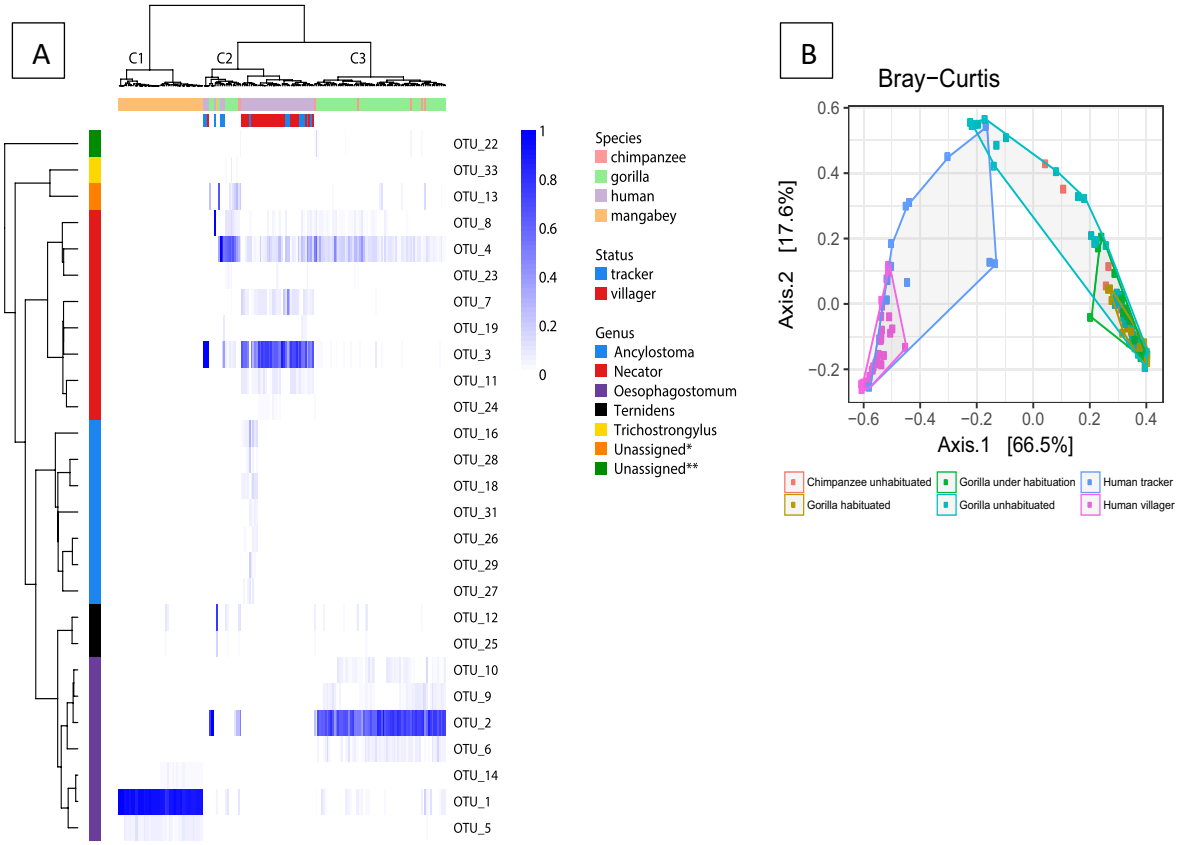


Figure 4. A) Heat map showing the relative abundances of dominant ITS-2 haplotypes (i.e., average relative abundance >0.1%) in individual samples. Columns were clustered using Ward algorithm. Clustering of rows reflects phylogenetic relationships among individual haplotypes. Column annotations indicate host species identity, whereas row annotation shows genus level identity and significant/nonsignificant abundance differences of a given haplotype among sampled hosts according to mvabund analyzes. *Closest sequence *Libyostrongylus* (probably *Paralibyostrongylus*) environmental sample, NCBI accession: JX159807.1 (100% identity), **closest sequences of *Nematodirus* (ca. 83% identity). B) PCoA for human and NHP samples. Bray-Curtis dissimilarities accounting for relative abundance of ITS-2 haplotypes. Ordination space occupied by different sample categories is indicated by convex Hull polygons. Pafčo et al., 2019.

The observed patterns suggest that host phylogeny alone cannot explain the overlap of parasite communities among host species. The more phylogenetically distant mangabeys harboured a strongylid community clearly distinct from those of humans and African great apes, consistent with differences in host compatibility. Yet, among hominids, gorilla and chimpanzee strongylid communities were more similar to each other than either was to the community in humans, despite humans being phylogenetically more closely related to chimpanzees than to gorillas. Ecological exposure therefore appeared to modify the pattern expected from host phylogeny.

The transmission biology of hookworms offers a possible explanation for their circulation among the studied primates. Infective *Necator* L3 penetrate the skin and therefore require contact with contaminated soil. Humans entering forest habitats may consequently encounter infective larvae originating from non-human primates without ever directly contacting an infected animal. This provides a plausible mechanism for the occurrence of *N. gorillae* in humans. Conversely, orally transmitted strongylids such as *Oesophagostomum* require ingestion of infective larvae, making their transmission

dependent on different patterns of environmental exposure, including consumption of contaminated plant material, soil or water. Thus, contact at the human–wildlife interface should not be understood simply as physical proximity between host species; its epidemiological significance depends on parasite transmission biology.

We further investigated these relationships in an unprotected area at the periphery of the Dja Faunal Reserve in Cameroon (Ilík et al., 2023). Here, western lowland gorillas, central chimpanzees and humans inhabit a landscape shaped by logging, hunting, agriculture and forest fragmentation. Using the same ITS-2 metabarcoding approach, we detected a highly diverse strongylid fauna (Figure 5). Great apes again harboured greater strongylid diversity than humans, and their communities were dominated by *N. gorillae*, *O. stephanostomum* and trichostrongylids, whereas humans were predominantly infected with *N. americanus*. Eight ASVs were shared between humans and great apes, including four variants assigned to *N. americanus*, two to *N. gorillae*, one *O. stephanostomum* type I and one *Trichostrongylus* variant (Figure 5).

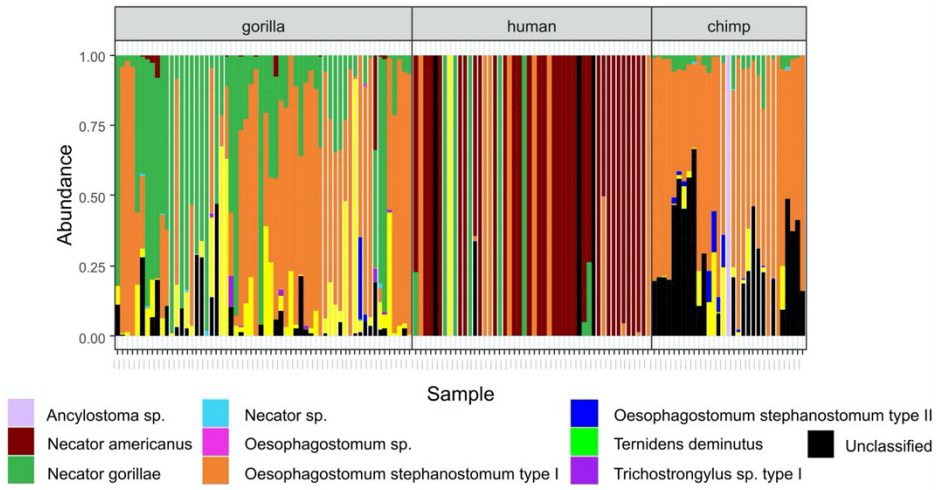


Figure 5. Bar plots showing relative community composition of strongylid nematodes at the species level in examined samples of gorilla, chimpanzees and humans at the periphery of Dja Faunal Reserve, Cameroon. Numbers of relative abundances of reads are depicted as colour panels. Ilík et al., 2023.

The Cameroonian study supported two observations from DSPA. First, parasites traditionally associated with particular host species were also detected in other hosts: *N. americanus* variants occurred in gorillas, whereas *N. gorillae* was detected in humans. Second, the extent of parasite sharing appeared to differ between the two ecological settings. More strongylid ASVs were shared between humans and great apes at Dja than in the protected DSPA system. The Dja landscape is characterised by intensive human use, with agricultural fields and degraded forest creating opportunities for humans and great apes to use the same areas. For soil-transmitted parasites, such ecological overlap may increase opportunities for transmission even without direct contact between hosts. Together, these findings suggest that host phylogeny alone cannot explain patterns of parasite sharing, as ecological exposure and parasite transmission biology also play important roles.

3.2 Different parasites, different patterns: the example of *Entamoeba*

The relative importance of host phylogeny and ecological exposure, however, is not the same across all gastrointestinal parasites. This became evident when we investigated *Entamoeba* communities in humans, western lowland gorillas and central chimpanzees from the Dja Faunal Reserve using 18S rDNA metabarcoding (Vlčková et al., 2018). As with strongylid nematodes, *Entamoeba* communities can comprise multiple species and genetically distinct lineages that are difficult or impossible to distinguish using conventional microscopy. We identified 36 haplotypes belonging to six major haplotype groups, including both apparently host-specific and more broadly distributed lineages. Most belonged to *Entamoeba* species generally considered commensal, although potentially pathogenic species, including *E. histolytica* and *E. nuttalli*, were also detected. Several haplotypes occurred in more than one host species, indicating potential for parasite sharing.

The overall pattern, however, differed markedly from that observed for strongylids. Whereas strongylid communities of gorillas and chimpanzees were relatively similar and humans formed a more distinct group, *Entamoeba* communities showed greater overlap between humans and chimpanzees, with gorillas being more distinct. In this case, community structure therefore corresponded more closely to host phylogeny (Vlčková et al., 2018). This comparison is particularly informative because the host populations and ecological setting were the same; what differed was the parasite group and its biology.

These findings illustrate that zoonotic potential cannot be inferred simply from the degree of spatial overlap between host species. Parasite traits, including transmission mode, environmental persistence and physiological requirements for establishment, interact with host characteristics to determine the outcome of exposure. Even within a single parasite genus, different lineages may show considerable variation in host specificity. Our *Entamoeba* study identified both broadly shared and strongly host-associated haplotypes within the same communities. Similarly, among strongylids, some *Necator* variants were shared among host species, whereas many other taxa remained strongly associated with particular hosts.

The *Entamoeba* results also illustrate another important distinction: parasite sharing does not necessarily imply similar health consequences across hosts. Most detected *Entamoeba* haplotypes belonged to species generally considered commensal, although potentially pathogenic species were also present. Establishing whether a parasite lineage can cross host boundaries is therefore only one aspect of assessing its zoonotic potential. Whether it can establish a persistent infection and cause disease in a new host must be considered separately.

3.3 *Strongyloides* as a multihost system

Strongyloides provides perhaps the clearest example of why parasite host specificity needs to be considered at the level of species and genetic lineages rather than genus alone. More than 50 *Strongyloides* species have been described, but the host ranges of individual species, where known, vary considerably. Among primate-associated species, *S. stercoralis* has a broad host range and occurs in

humans, dogs and non-human primates, whereas *S. fuelleborni* is primarily associated with African and Asian non-human primates but can also infect humans. Our review of *Strongyloides* infections in non-human primates showed, however, that understanding host specificity remains constrained by substantial gaps in available data (Nosková et al., 2024b). Many studies identify infections only to genus level, and molecular information is geographically and taxonomically uneven (Figure 6A). Dogs are particularly underrepresented despite their potential role as bridge hosts between humans and non-human primates (Figure 6B). We therefore highlighted the need for standardised molecular identification across humans, non-human primates and dogs to better understand parasite circulation and inform public-health control strategies.

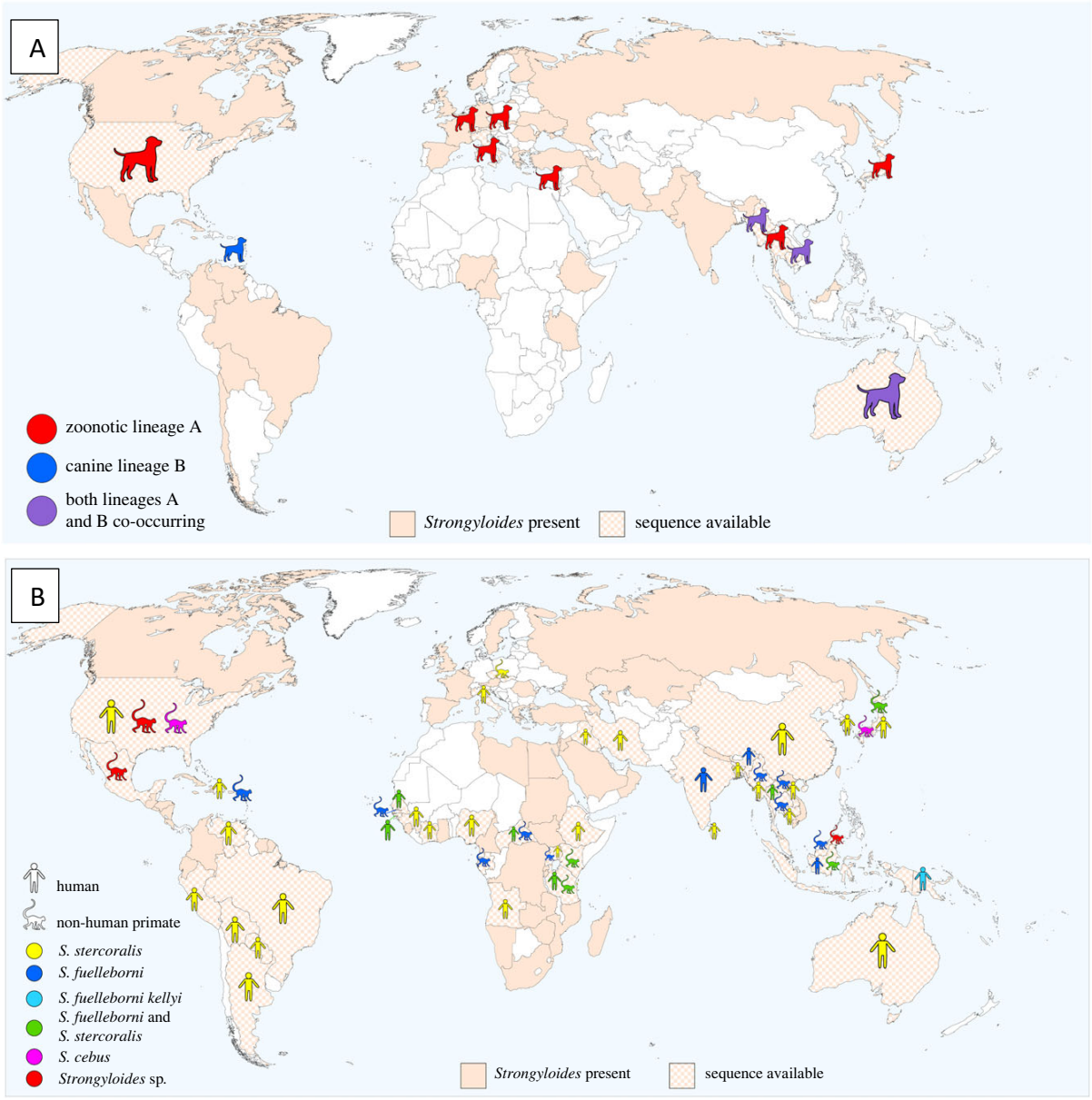


Figure 6. A) Geographical distribution of *Strongyloides* spp. in primates, including humans. Chequered filling marks the countries from which any DNA sequences are available in GenBank, together with the host's (human/non-human primate) and the parasite's identity. **B)** *Strongyloides stercoralis* in dogs. For chequered-filled countries, DNA sequences are available in GenBank. Nosková et al., 2024b.

Differences in *Strongyloides* species reported from wild and captive primates add another dimension to our understanding of host specificity. *Strongyloides fuelleborni* is frequently associated with free-ranging Old World primates, whereas *S. stercoralis* is more commonly reported from captive non-human primates, where proximity to humans and domestic animals may facilitate exposure (Nosková et al., 2024b). We demonstrated the presence of *S. stercoralis* in captive orangutans from Czech and Slovak zoological gardens (Nosková et al., 2023). Molecular analysis of *Strongyloides* larvae identified *S. stercoralis* belonging to cox1 lineage A, which is also found in humans, dogs and other primates. Captive environments represent an extreme example of altered host contact. Humans, primates and sometimes other animals occupy restricted environments, potentially increasing opportunities for parasites with broad host specificity to circulate among species. The detection of a zoonotic lineage of *S. stercoralis* in captive orangutans therefore raises concerns about possible transmission between primates and zoo personnel. Although the direction and extent of transmission remain unknown, these findings highlight the importance of molecular surveillance and appropriate preventive measures in zoological collections.

3.4 From two-host systems to multihost transmission networks

A major limitation of many studies on zoonotic parasites is their focus on a single presumed reservoir and a single recipient host. However, natural transmission systems are often considerably more complex. Humans, wildlife, domestic animals and environmental stages may form interconnected networks, and excluding one host group can lead to incomplete conclusions about potential sources and reservoirs of infection. Our work demonstrates why a One Health perspective is particularly important in such systems.

This has direct relevance for *Strongyloides*, which is now included among the soil-transmitted helminths targeted by WHO control programmes. In 2024, WHO issued its first guideline for the public-health control of strongyloidiasis, recommending annual mass drug administration with ivermectin in endemic settings where *S. stercoralis* prevalence exceeds a defined threshold (WHO, 2024). However, several *Strongyloides* species and genetic lineages have been detected across humans, non-human primates and dogs living in the same environments (Nosková et al., 2024b). If animals contribute to the maintenance and environmental circulation of lineages capable of infecting humans, interventions targeting exclusively human populations may not be sufficient to interrupt transmission. Wildlife populations cannot realistically be included in routine treatment programmes, making it particularly important to understand the host range of individual parasite lineages and the relative contribution of different hosts to their transmission.

We investigated these questions in our recent study of *Strongyloides* in the Dzanga-Sangha Protected Areas (Nosková et al., 2026), returning to the same Central African ecosystem examined in our earlier strongylid research but extending sampling to humans, non-human primates and domestic dogs. Using qPCR followed by high-throughput sequencing of the 18S rDNA HVR-IV and cox1

regions, we detected a high prevalence of *Strongyloides* in humans, dogs and gorillas, together with substantial genetic diversity within the genus. *Strongyloides stercoralis* haplotype A occurred predominantly in humans but was also detected in dogs and gorillas, whereas *S. fuelleborni* was detected across several host groups.

The multihost design provided a more complex epidemiological picture. *Strongyloides* communities were more similar between humans and dogs than between either of these groups and non-human primates, and the largest proportion of shared haplotypes occurred between humans and dogs (Figure 7). At the same time, several *S. fuelleborni* haplotypes were shared among humans, non-human primates and dogs (Figure 7). This was particularly interesting because *S. fuelleborni* has traditionally been regarded primarily as a parasite of non-human primates, with human infections also reported. The detection of shared haplotypes in dogs raises the possibility that they may contribute to parasite circulation between human settlements and surrounding wildlife habitats, although their role as competent hosts and potential reservoirs requires further investigation.

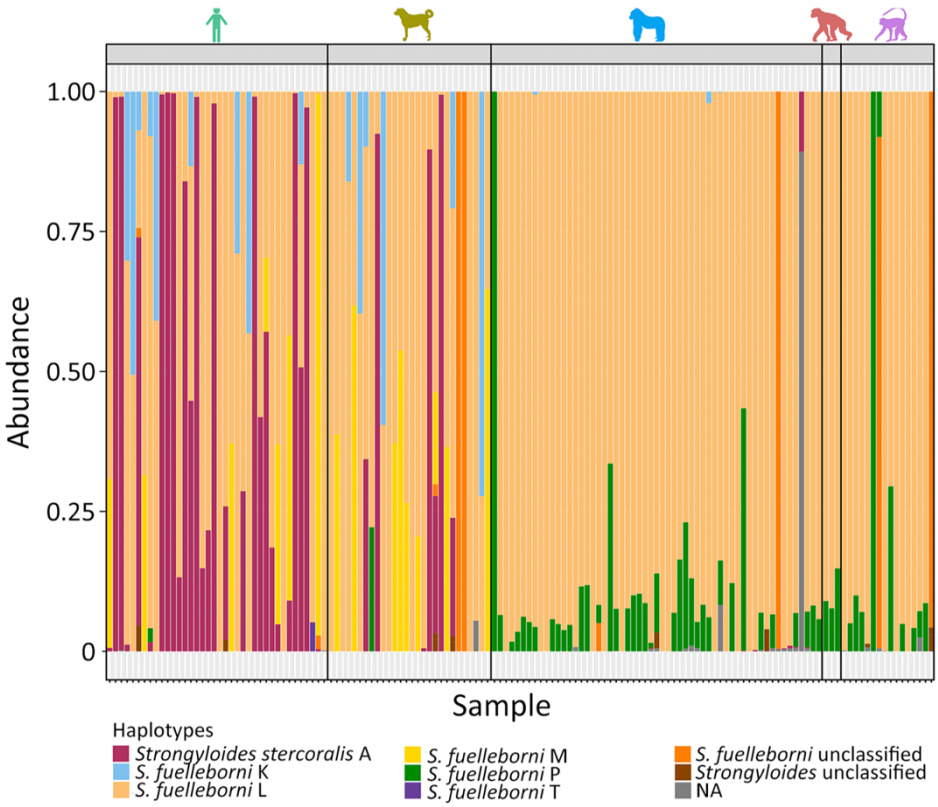


Figure 7. Relative community composition of *Strongyloides* HVR-IV-18S rRNA haplotypes across examined hosts in study of *Strongyloides* genetic diversity among humans, dogs, and nonhuman primates, Dzanga-Sangha Protected Areas, Central African Republic, 2016–2022. Relative abundance of haplotypes shown as colour panels; each column represents a single sample. Nosková et al., 2026.

In DSPA, humans, dogs and non-human primates share several *S. stercoralis* and *S. fuelleborni* haplotypes, while the free-living stages of *Strongyloides* develop in soil. The epidemiological system therefore comprises not only multiple vertebrate hosts but also a shared environmental component. This has potential consequences for control: treating infected humans alone may not interrupt transmission if infective stages remain in the environment and the same parasite lineages are maintained in domestic

animals or wildlife. The CAR study therefore provides a particularly clear example of why a One Health approach is not merely conceptual but arises directly from parasite biology.

A similar multihost perspective was applied in the Bwindi ecosystem, Uganda, where we investigated *Strongyloides* in mountain gorillas, olive baboons and domestic animals (Bukamba et al., 2026). Molecular analyses identified identical *S. fuelleborni* HVR-IV sequences in mountain gorillas and baboons, which also matched sequences previously reported from other African primates and humans. In domestic animals, *S. ransomi* was detected in pigs, while a potentially zoonotic *S. stercoralis* lineage A genotype was identified in a dog. Interestingly, *Strongyloides* prevalence was higher in gorilla groups ranging near the forest edge than in those inhabiting the forest interior. Although these findings do not demonstrate direct cross-species transmission, they provide further evidence of overlapping parasite host ranges and highlight the potential importance of shared environments for *Strongyloides* circulation at the wildlife–human interface.

The importance of considering multiple sympatric hosts is also demonstrated by our study at Lake Nabugabo, Uganda (Upadhayay et al., 2026). Here, parasite communities were compared among vervet monkeys, humans, represented by household latrine samples, dogs and livestock. Across these hosts, 33 parasite taxa were identified using microscopy together with molecular characterisation of strongylids, *Strongyloides* and selected protists. Parasite communities differed substantially among host groups, and overall overlap between vervets and the other hosts was limited. Nevertheless, particular parasites crossed these apparent community boundaries. Most notably, *Giardia intestinalis* assemblage A was detected in vervets, dogs and human latrine samples, whereas several strongylid ASVs were shared across combinations of domestic animals, humans and vervets.

Dogs repeatedly emerge as particularly interesting components of these multihost systems. At Lake Nabugabo, they harboured the greatest number of parasite taxa among the sampled host groups and shared parasites with humans, livestock and wildlife. Free-ranging dogs move between human settlements and surrounding environments, scavenge and exhibit coprophagy, creating opportunities both for true infections and for mechanical passage of parasite stages. Similarly, in CAR, dogs occur at the interface between villages and forests, particularly when accompanying humans during hunting. Their epidemiological importance may therefore derive not only from their capacity to act as definitive hosts, but also from their behaviour and mobility across otherwise partially separated ecological compartments.

4. Ecological and anthropogenic drivers of parasite communities

Parasite communities are not static attributes of host species. Even when a host is susceptible to a particular parasite, whether infection occurs and at what intensity depends on a combination of environmental conditions, host ecology and behaviour, population structure and opportunities for transmission. For parasites with environmental life stages, these factors are particularly important because temperature, humidity, vegetation and patterns of habitat use affect the survival and distribution

of infective stages outside the host. At the same time, host age, individual immunity, social organisation, population density, diet and ranging behaviour influence both exposure and susceptibility. Anthropogenic change can modify many of these factors simultaneously, making its effects on parasite communities difficult to predict (Faust et al., 2018; Gibb et al., 2020).

Importantly, anthropogenic disturbance should not be expected simply to increase the intensity or diversity of parasite infections. Human activities can increase contact among humans, domestic animals and wildlife, alter host density and behaviour, change resource availability and induce physiological stress, all of which may increase exposure or susceptibility (Hassell et al., 2017; Faust et al., 2018). Conversely, habitat fragmentation and environmental degradation can disrupt parasite transmission cycles and lead to the loss of components of the natural gastrointestinal community. This distinction is particularly relevant because gastrointestinal organisms encompass a continuum from pathogens through commensals to organisms that may contribute to normal gut function. Thus, loss of parasite or symbiont diversity cannot automatically be interpreted as an improvement in host health.

Our studies across several African primate systems illustrate this complexity. Rather than revealing a uniform effect of human disturbance, they show that gastrointestinal parasite communities are shaped by interactions among host ecology, habitat characteristics, social organisation and anthropogenic pressure, and that the relative importance of these factors varies among host and parasite taxa. This complexity also presents a challenge for the increasing use of wildlife health monitoring as a component of conservation programmes, highlighting the need to understand the ecological processes underlying observed patterns of parasite infection.

4.1 Habitat disturbance and loss of gastrointestinal symbiont diversity

Comparison of gastrointestinal symbionts in two sympatric primates, the Udzungwa red colobus (*Procolobus gordonorum*) and yellow baboon (*Papio cynocephalus*), living in protected and unprotected forests in the Udzungwa Mountains of Tanzania was an important starting point for our research in this field (Barelli et al., 2020). The two species differ substantially in their ecology: red colobus are predominantly arboreal and folivorous, whereas yellow baboons are terrestrial dietary generalists that frequently exploit crops and human food resources. This provided an opportunity to separate, at least partly, the effects of habitat disturbance from those of host ecology.

The two host species differed strongly in their gastrointestinal symbiont richness. Yellow baboons harboured substantially greater richness than red colobus in both forest types. However, habitat disturbance affected the two hosts differently (Figure 8). Red colobus inhabiting the continuous protected Mwanihana forest had almost twice the gut symbiont richness of those living in the unprotected Magombera forest fragment. In contrast, no significant difference between protected and unprotected forests was observed in opportunistically foraging yellow baboons (Barelli et al., 2020).

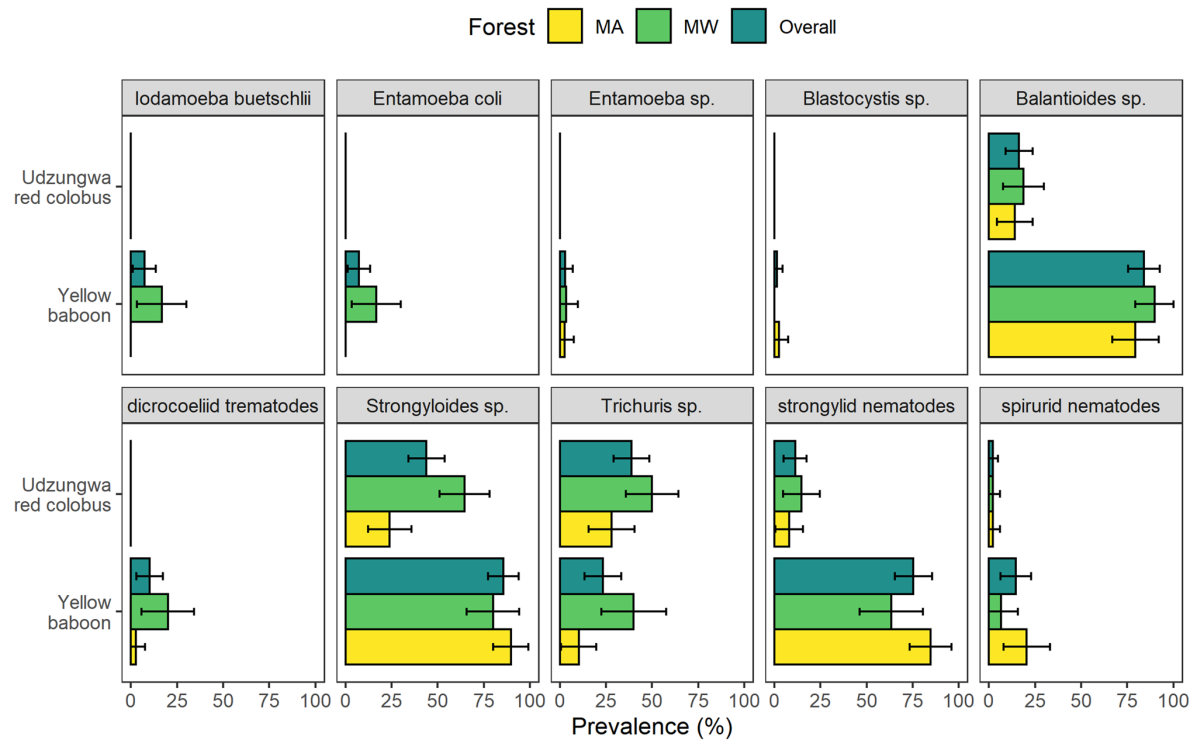


Figure 8. Mean prevalence (expressed in %) of intestinal protozoan and metazoan symbionts in two primate species: Udzungwa red colobus (*Procolobus gordonorum*) and yellow baboon (*Papio cynocephalus*). Colored bars represent the mean symbiont prevalence found from fecal samples of red colobus (N = 69) and yellow baboons (N = 69) collected in two forest types within the Udzungwa Mountains of Tanzania: the unprotected Magombera (MA, colored in yellow) and the protected Mwanihana (MW, light green) forests. Dark green bars indicate the overall mean prevalence for both forest types. Horizontal bars represent the 95% confidence interval. Barelli et al., 2020.

These results demonstrated two important principles. First, host ecology can be at least as important as habitat itself in determining gastrointestinal community structure. The greater diversity observed in baboons is consistent with their terrestrial lifestyle and broader diet, which expose them to a wider range of environmentally and trophically transmitted organisms. Spirurid nematodes and dicrocoeliid trematodes, for example, require intermediate hosts, and terrestrial omnivorous baboons are more likely to ingest the arthropods involved in these transmission cycles than predominantly folivorous, arboreal red colobus. Second, the same anthropogenic alteration can have different consequences for different host species. The decline in symbiont richness in red colobus paralleled previously observed reductions in their bacterial gut diversity, whereas the more ecologically flexible baboons appeared comparatively resilient. This suggests that the sensitivity of the gastrointestinal ecosystem to environmental disturbance depends partly on the ecological flexibility of the host.

The direction of the observed change is also important. Rather than acquiring more gastrointestinal organisms in disturbed habitat, red colobus lost symbiont diversity (Barelli et al., 2020). This finding challenges the traditional assumption that parasites should primarily be considered harmful organisms whose disappearance would benefit wildlife. Protozoan and metazoan gut inhabitants participate in long-standing ecological and evolutionary interactions with their hosts, and at least some may have neutral or beneficial effects. Habitat degradation may therefore cause a form of internal biodiversity loss alongside the much more visible loss of environmental biodiversity. In this sense,

conservation of a host species may also require consideration of its associated gastrointestinal community. The red-colobus study demonstrated that human activities can affect this "microbiodiversity" even when the host itself persists in the disturbed habitat.

4.2 Human disturbance does not have a uniform effect on parasite communities

We subsequently examined yellow baboons across a more explicit gradient of anthropogenic disturbance in western Tanzania (Mason et al., 2022b). Nine troops inhabited areas differing in the intensity of tree cutting, livestock grazing, human pathways and proximity to settlements, allowing parasite patterns to be evaluated against measured variation in human disturbance rather than simply comparing protected and unprotected habitats.

The results did not reveal a simple increase in parasitism with disturbance. Eleven gastrointestinal taxa (seven protozoan and four helminth taxa) were identified, but individual taxa responded differently. *Entamoeba coli* and *Iodamoeba buetschlii* were more prevalent in less disturbed areas, whereas increasing disturbance was associated with greater abundance of unidentified isotrichid ciliates (Figure 9A). Strongylids occurred in all samples, but their abundance did not differ significantly with disturbance (Figure 9B). At the same time, the prevalence of several parasite taxa was associated with baboon group size, and *E. coli* and *Strongyloides* infections were more prevalent in juveniles.

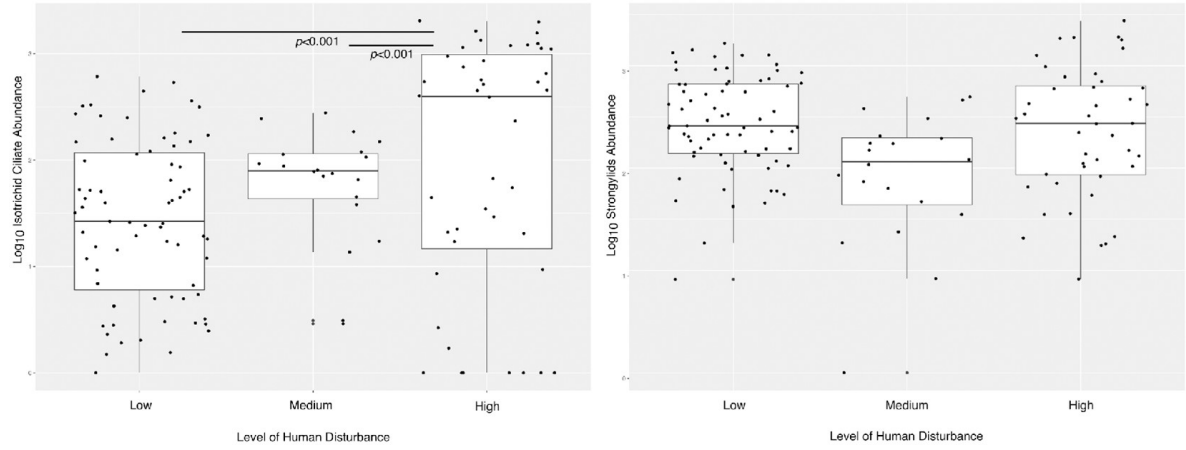


Figure 9. Parasite abundance in yellow baboons in western Tanzania. Plots are split by human disturbance of habitat area and show two parasite taxa: (A) unidentified isotrichid ciliate; (B) strongylids. Mason et al., 2022b.

This mixed response is informative. Human disturbance can influence parasite infections through several, potentially opposing pathways. Greater contact with humans and livestock can introduce new opportunities for cross-species transmission (Faust et al., 2018). Habitat modification can alter ranging and feeding behaviour and therefore exposure to environmental infective stages. Reduced food availability may influence host nutritional status, while repeated human disturbance may affect physiological stress and immunity. Conversely, environmental degradation can remove intermediate hosts or alter microclimatic conditions required for parasite development, thereby reducing transmission. The net outcome will therefore depend on the biology of individual parasite taxa and on the ecology of the host.

The baboon studies are particularly useful in demonstrating this context dependence. As terrestrial dietary generalists, baboons readily exploit human-modified landscapes and may therefore respond very differently to habitat disturbance than more specialised primates such as red colobus. Consequently, "human disturbance" itself is not a biological mechanism. It represents a collection of environmental changes whose effects are mediated through host behaviour, host population structure and parasite life history.

4.3 Ecotourism, physiological stress and parasite infection

Anthropogenic effects may also operate through host physiology rather than through changes in habitat or direct parasite exposure. Wildlife tourism provides a particularly interesting example because habituation to humans can simultaneously benefit conservation and expose animals to a novel chronic stressor. In western lowland gorillas in Dzanga-Sangha Protected Areas (DSPA), we therefore examined whether physiological changes associated with habituation and ecotourism were related to strongylid infection intensity (Shutt-Phillips et al., 2021).

Our previous work had shown that gorillas undergoing habituation had higher faecal glucocorticoid metabolite (FGCM) concentrations than fully habituated and unhabituated gorillas (Shutt et al., 2014). Chronically elevated glucocorticoids can affect immune function and could consequently reduce the host's ability to regulate parasite infections. Here we therefore tested whether FGCM concentrations were associated with faecal egg counts of *Necator/Oesophagostomum* and *Mammomonogamus*, and whether infection intensities differed among gorillas experiencing different levels of habituation (Shutt-Phillips et al., 2021). The results again emphasised that the relationship was not straightforward. Within the habituated Makumba group and the Mata group undergoing habituation, we found no significant direct relationship between FGCM concentrations and strongylid egg counts. However, comparisons among groups revealed significant differences: *Necator/Oesophagostomum* egg counts were higher in the group undergoing habituation and in one fully habituated group than in unhabituated gorillas. These group-level patterns broadly paralleled differences in glucocorticoid levels. Season and age also influenced strongylid infection intensity.

The study therefore provided only limited support for a direct glucocorticoid–parasite relationship and should not be interpreted as demonstrating that ecotourism causes increased parasite infection. This is consistent with the broader literature, which has revealed considerable variation in the relationships between glucocorticoids and parasitism across mammalian species (Defolie et al., 2020). Nevertheless, our study showed that parasite intensity can vary among animals exposed to different degrees of human contact and physiological stress. Both FGCM concentrations and parasite intensities were generally lower in unhabituated gorillas than in groups undergoing habituation or already habituated, although the limited number of groups makes causal interpretation difficult.

Human presence may influence parasite infections indirectly through stress-mediated changes in immunity, but habituation may also alter ranging patterns, habitat use and exposure to infective stages.

Distinguishing these mechanisms requires considering physiological responses alongside the broader ecological conditions in which hosts and parasites interact.

4.4 Spatial heterogeneity in mountain-gorilla helminth infections

The importance of addressing multiple ecological drivers in parasite ecology became particularly clear in our studies on mountain gorillas. These iconic and endangered apes provide an unusual system because conservation success has resulted in population growth within a geographically restricted habitat. Their two remaining populations are confined to the Virunga Massif and Bwindi–Sarambwe ecosystems, which are surrounded by densely populated human landscapes and offer little opportunity for spatial expansion. In parts of the Virunga Massif, population growth has consequently been accompanied by increased gorilla density, changes in group structure, greater home-range overlap and increased rates of intergroup encounters (Caillaud et al., 2020).

A range-wide comparison among mountain-gorilla populations and subpopulations revealed striking heterogeneity in strongylid and tapeworm infections (Petrželková et al., 2021). Strongylid egg counts differed substantially across geographical areas, with the highest values recorded in Bwindi, followed by parts of the Virunga Massif where gastrointestinal disease had also been observed. Within Virunga, infection intensity differed among areas, and relationships with group size were themselves geographically variable. Host age and season also contributed to variation in helminth egg counts.

The major implication was that mountain gorillas cannot be treated as a homogeneous host population with a characteristic level of helminth infection. Even within this single subspecies, strongylid infection intensity varies substantially across a relatively restricted geographical range. The spatial patterns potentially reflected several correlated processes, including differences in vegetation and elevation, historical population growth, gorilla density and changes in social organisation. Although this first range-wide study identified substantial heterogeneity, we were unable to fully disentangle its drivers because geographical area represented several ecological variables simultaneously.

These difficulties led directly to the subsequent Virunga-wide analysis, in which we explicitly evaluated environmental and host factors using samples collected during the 2015–2016 population surveys (Petrželková et al., 2022). Importantly, these surveys included both monitored/habituated and unmonitored/unhabituated groups, allowing the effects of habituation to be considered alongside group size, relative gorilla density, the area represented by buffered nest-site polygons and environmental variables including elevation, temperature, precipitation and vegetation. In this case, strongylid egg counts were higher in groups with smaller areas represented by buffered nest-site polygons, at higher relative gorilla densities and in environments characterised by higher elevations, greater precipitation and lower temperatures. Vegetation types associated with these environmental conditions may provide favourable microclimates for the development, survival and migration of infective strongylid L3 larvae. Conversely, groups distributed across larger areas may experience reduced exposure to contaminated sites, although the buffered nest-site polygons used in our analysis were not direct estimates of home

ranges. In contrast to the group-level differences observed in western lowland gorillas (Shutt-Phillips et al., 2021), the effect of habituation in the Virunga mountain-gorilla population was comparatively minor and was primarily detected for tapeworm infections in interaction with environmental variables.

These results are particularly interesting because some of the ecological conditions associated with higher infection are themselves partly consequences of the history of mountain-gorilla conservation and habitat change. The recovery of the Virunga population has increased gorilla numbers within a habitat whose boundaries cannot readily expand. Moreover, historical deforestation removed much of the lower-elevation forest in Rwanda, restricting part of the population to higher elevations. Thus, contemporary parasite epidemiology reflects not only immediate environmental conditions but also the longer-term interaction between anthropogenic habitat modification and successful conservation. At the same time, environmental effects may operate on both sides of the host–parasite relationship. Cooler, wetter high-elevation habitats may favour survival of free-living strongylid stages, while differences in vegetation and diet may influence gorilla nutrition and susceptibility. The available data cannot completely separate these mechanisms, and experimental work on larval development and survival across mountain-gorilla habitats would be needed to determine their relative importance.

5. From infection to disease: health consequences of gastrointestinal parasites in wild primates

The presence of a parasite does not necessarily imply disease. Wild primates harbour diverse gastrointestinal eukaryotic communities, many of which represent long-standing host–parasite associations and persist without apparent clinical consequences. Even within a single host population, infections can range from apparently asymptomatic carriage to severe morbidity and mortality. Understanding parasite-associated disease therefore requires moving beyond parasite detection and prevalence to identify which parasites are associated with pathology, under what conditions infections become clinically significant, and how changes in host or environmental conditions may influence disease outcomes.

Mountain gorillas provide an unusual opportunity to address these questions. Their gastrointestinal parasites have been monitored over several decades, while intensive veterinary surveillance provides information rarely available for free-ranging wildlife, including clinical histories, treatment responses, necropsies and histopathology (Muhangi et al., 2021). At the same time, the two remaining mountain gorilla populations are geographically isolated and have undergone substantial demographic and ecological changes. Reports of severe gastrointestinal disease therefore raise important questions about the roles of parasite identity and abundance, host condition and environmental factors in determining clinical outcomes.

5.1 Parasite presence and infection intensity do not necessarily indicate disease

A useful contrast is provided by anoplocephalid tapeworm infections. Mountain gorillas commonly harbour *Anoplocephala gorillae*, together with less frequent *Bertiella* infections. Our investigation

combined examination of adult cestodes recovered during necropsies, proglottids in faeces, night-nest faecal samples and longitudinal samples from identified individuals, together with morphological and molecular characterisation (Červená et al., 2024). *Anoplocephala gorillae* was the dominant cestode, and individual gorillas showed considerable short-term fluctuations in egg shedding. These fluctuations complicate the interpretation of faecal egg counts, as a single high count does not necessarily indicate a persistently high level of egg output or a correspondingly high worm burden. Importantly, despite its frequent occurrence, there is currently no clear evidence that *A. gorillae* causes significant gastrointestinal disease in mountain gorillas.

A similar distinction between parasite detection and disease emerged from our investigation of *Cryptosporidium*, *Giardia* and microsporidia across the Virunga Massif (Uwamahoro et al., 2025). Although *Giardia intestinalis* and *Enterocytozoon bieneusi* were detected, their distribution and temporal patterns did not correspond with the observed gastrointestinal disease. These findings suggest that the investigated protists were unlikely to explain the increasing occurrence of severe gastrointestinal problems in Virunga gorillas.

Together, these studies illustrate why the presence of gastrointestinal parasites should not automatically be interpreted as evidence of pathogenicity and provide a useful contrast to the strongylid-associated disease discussed below.

5.2 Emergence of pathogenic strongylids in the Virunga Massif

The clearest evidence comes from the Virunga Massif, where severe and sometimes fatal gastritis has occurred disproportionately in gorilla groups ranging between Mount Karisimbi and Mount Bisoke. During the most intense period, 2008–2016, up to 25 fatal gastritis cases were recorded, with approximately 84% concentrated in this region. Histopathological examination demonstrated gastric lesions associated with trichostrongylid nematodes. The fact that gastric trichostrongyles were not detected histologically in animals without gastritis further supports their association with the observed clinical syndrome (Muhangi et al., 2021).

Our earlier coprological studies revealed substantial geographical variation in strongylid egg counts, including higher counts in areas where severe gastritis occurred more frequently (Petrželková et al., 2021, 2022). Metabarcoding subsequently allowed us to move beyond the morphologically limited category of “strongylids” and examine the composition of these complex nematode communities. By combining metabarcoding data with faecal egg counts, we could estimate the egg output attributable to individual strongylid taxa rather than relying exclusively on relative sequence abundance. This identified *Hyostrongylus*, a genus of gastric strongylids known to be pathogenic in other mammalian hosts (especially suids), as the principal taxon associated with the gastric lesions (Figure 10). The highest estimated egg output attributable to *Hyostrongylus* occurred in the high-altitude region in which severe gastritis was concentrated (Pafčo et al., 2026).

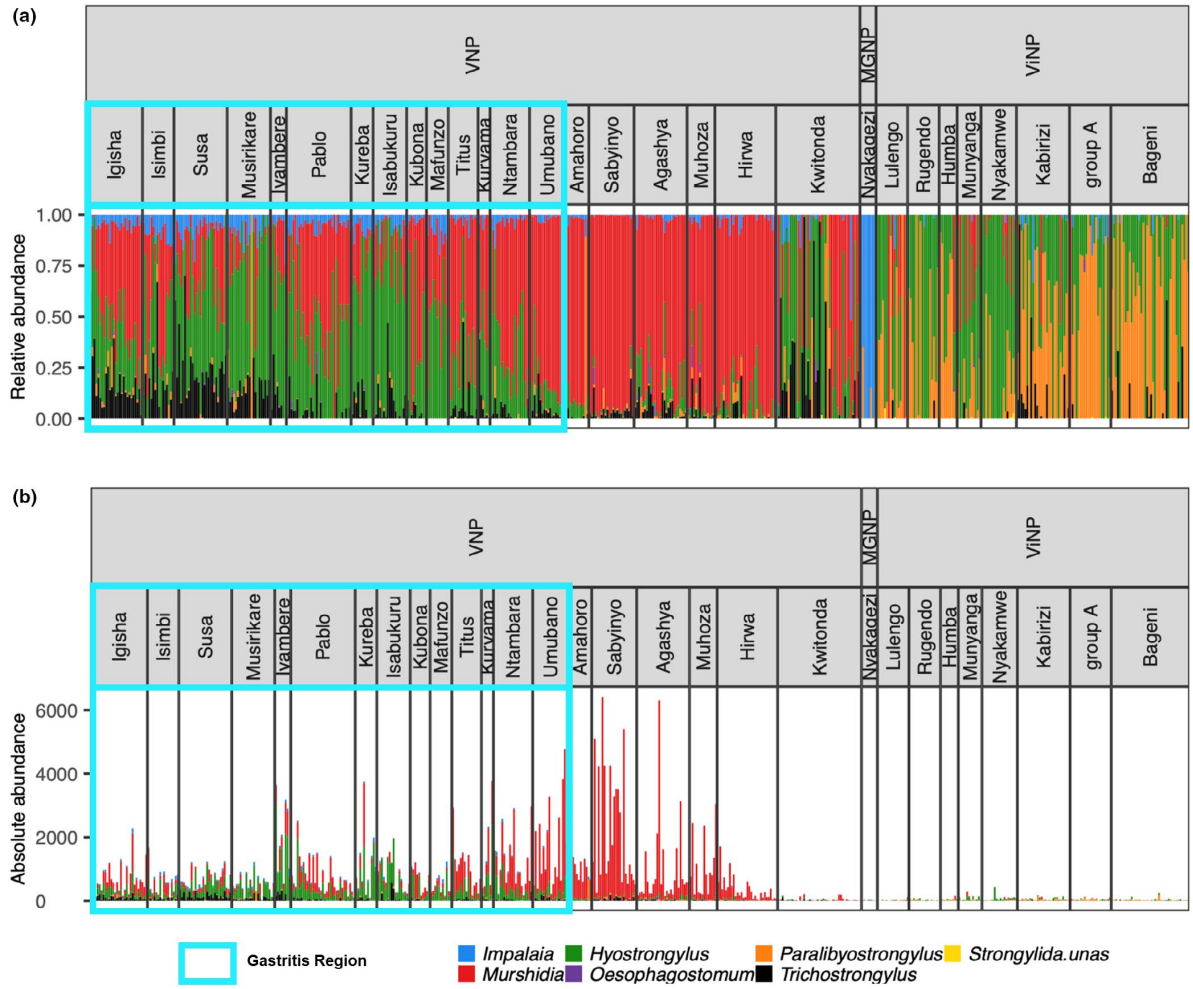


Figure 10. Bar plot showing the (a) relative abundance and (b) absolute abundance of strongylid genera in studied mountain gorilla groups inhabiting the Virunga Massif (Volcanoes National Park in northwestern Rwanda, Mgahinga Gorilla National Park in southwestern Uganda and Virunga National Park in eastern Democratic Republic of the Congo). The gastritis region, indicated by the blue box, highlights gorilla groups ranging within a single region where the majority (84%) of severe gastritis cases occurred. Pafčo et al., 2026.

These findings change the interpretation of strongylid nematode presence in mountain gorillas during health monitoring. Strongylids are widespread components of the gastrointestinal communities of wild African great apes, and their presence itself is not necessarily associated with disease. Indeed, strongylid communities in western lowland gorillas are dominated by taxa such as *Necator* and *Oesophagostomum*, with no comparable reports of fatal strongylid-associated disease (Mason et al., 2022a). Thus, the relevant question is not why mountain gorillas harbour strongylids, but why particular components of the strongylid community are associated with pathology in a particular host subpopulation.

The geographical pattern of observed clinical cases provides part of the answer. As discussed in Chapter 4, strongylid communities in the Virunga Massif vary along environmental gradients. The abundance of the potentially pathogenic *Hyostrongylus* is concentrated in cooler, wetter, high-altitude areas (Pafčo et al., 2026). These environmental conditions may favour the survival and transmission of infective larvae, while gorilla population growth within a spatially restricted habitat may have increased opportunities for parasite transmission. An additional factor may be the evolutionary history of the host–

parasite association. *Hyostrongylus* is absent or extremely rare in other African great ape populations examined in our studies, including western lowland gorillas and chimpanzees, whereas members of this genus are typically associated with suids. Its occurrence in mountain gorillas may therefore represent a relatively recent host association, potentially lacking the long history of host–parasite coevolution characteristic of its usual hosts. Whether this contributes to the unusually severe pathology observed in mountain gorillas remains unknown, but it provides a hypothesis for future investigation. Disease emergence may thus reflect an interaction between parasite identity and abundance, environmental conditions, host ecology and the evolutionary history of the host–parasite relationship. The low genetic diversity resulting from historical population bottlenecks could further contribute to host vulnerability, although this remains difficult to demonstrate.

5.3 Historical samples reveal a changing host–parasite relationship

Contemporary associations between parasite abundance and disease cannot determine whether a pathogenic parasite has recently emerged or has been present in the host population for much longer. Addressing this question is particularly difficult in wildlife, for which longitudinal biological material is rarely available. Mountain gorillas represent an unusual exception: habituated groups have been monitored almost daily for decades, generating detailed long-term health and demographic records together with archived biological samples. This unique resource allowed us to reconstruct strongylid communities from archived faecal samples spanning approximately two decades and to investigate whether the parasite patterns observed today reflect recent emergence or longer-term changes in an established host–parasite system (Mason et al., 2025).

Most importantly, the dominance of *Hyostrongylus* in the Karisimbi–Bisoke (KB) region, where gastritis occurs more frequently, was not stable over time (Figure 11). *Hyostrongylus* was substantially less abundant in samples collected in 2001 than in contemporary samples, indicating a major shift in parasite community composition over the intervening two decades, during which gastrointestinal disease also became increasingly prominent (Mason et al., 2025). This temporal perspective is important because it suggests that the present disease pattern is not simply an intrinsic property of the mountain-gorilla–*Hyostrongylus* association. Instead, an existing host–parasite system appears to have changed. However, the factors responsible for the observed increase in *Hyostrongylus* abundance, and their contribution to gastrointestinal disease, remain unclear.

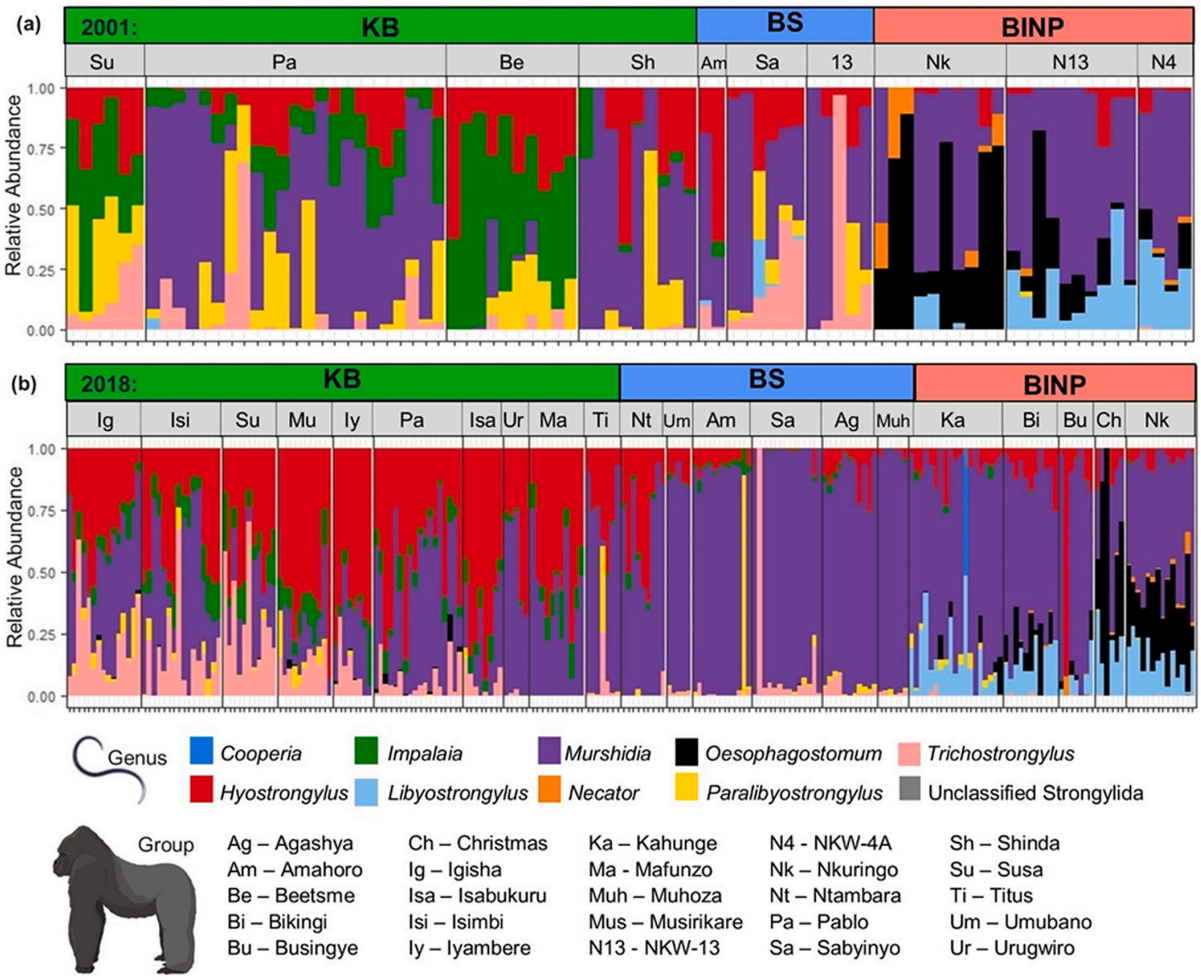


Figure 11. Community composition of strongylid nematodes of mountain gorillas sampled during two time periods: (a) 2001 and (b) 2018. Each column represents the relative abundances of ITS-2 amplicon sequence variants per sample with samples grouped by host group identity (lower x-axis) and ordered by region (upper x-axis): Karisimbi-Bisoke (KB), Bisoke-Sabyinyo (BS) – both situated in Volcanoes National Park – and Bwindi Impenetrable National Park (BINP). Mason et al., 2025.

5.4 A different strongylid-associated syndrome in Bwindi

The second mountain gorilla population, inhabiting Bwindi–Sarambwe, provides an important comparison. Interestingly, both mountain gorilla populations experience serious gastrointestinal health problems, in contrast to western lowland gorillas, for which comparable disease has not been reported. However, the clinical manifestations differ: while severe and sometimes fatal gastritis occurs in the Virunga Massif, the major emerging gastrointestinal health problem in Bwindi is chronic wasting (CW), characterised by progressive loss of body condition, lethargy, poor hair coat, browning, alopecia and, in severe cases, death. High nematode burdens have been observed in affected animals, and some individuals showed marked improvement following anthelmintic treatment, suggesting a possible role of intestinal helminths (Muhangi et al., 2021; Sambucci et al., 2026).

However, the molecular signature differs from the Virunga gastritis syndrome. We compared strongylid and bacterial communities in affected and unaffected Bwindi gorilla groups and identified *Oesophagostomum* as the principal taxon associated with chronic wasting. Groups with higher chronic wasting prevalence differed in strongylid community composition, whereas their bacterial communities

remained comparatively stable. Habituated gorillas also showed lower strongylid haplotype richness, greater relative abundance of *Oesophagostomum* and lower abundance of *Murshidia* than unhabituated gorillas (Sambucci et al., 2026).

This is particularly interesting because *Oesophagostomum* is not a newly introduced parasite. It is a common member of the gastrointestinal parasite communities of African great apes (Mason et al., 2022a). Consequently, its association with disease does not necessarily mean that the parasite alone is sufficient to cause chronic wasting. As we explicitly concluded, the higher abundance of *Oesophagostomum* could reflect a causal relationship, but alternatively, host genetic, immunological or environmental changes could allow a normally tolerated member of the gut community to proliferate (Sambucci et al., 2026). On the other hand, *Oesophagostomum* has well-documented pathogenic potential in primates (Terio et al., 2018; Jaffe et al., 2024). Pathological examination of mountain gorillas has also identified lesions associated with nodular worms, although their overall contribution to morbidity remains unclear (Muhangi et al., 2021). Thus, the long-term presence of *Oesophagostomum* in a host population does not preclude an important role in disease, although the factors determining its pathogenic effects in Bwindi gorillas remain unresolved.

The contrasting gastrointestinal syndromes observed in the two mountain gorilla populations illustrate the complexity of parasite-associated disease in wild primates. Although both populations harbour diverse strongylid communities, different parasite taxa are associated with their major clinical problems, and the factors determining these differences remain poorly understood. These findings highlight the importance of combining molecular identification of parasites with clinical and pathological observations to better understand their potential health consequences.

6. Synthesis and perspectives: towards an integrated understanding of host–parasite dynamics

The studies presented in this habilitation have addressed gastrointestinal parasites from several perspectives, ranging from their taxonomic and genetic diversity to host specificity, transmission ecology and consequences for host health. Although these questions were investigated in different host species and ecological settings, they are closely interconnected. Understanding which parasites occur in a particular host is only the first step. We also need to understand why they occur there, how they circulate among hosts and environments, and under which conditions they may become pathogenic.

An important part of my research has been the application of molecular approaches to parasite groups whose diversity is difficult to resolve using conventional methods. Metabarcoding has substantially improved our ability to characterise complex infections and compare parasite communities across hosts and environments. However, our studies have also demonstrated the limitations of interpreting sequence data without sufficient taxonomic references and biological information. The identification of identical or closely related parasite sequences in different hosts does not necessarily

demonstrate transmission, just as the detection of a parasite does not establish its contribution to disease. These distinctions have been particularly important when investigating the zoonotic potential of gastrointestinal parasites and their possible role in the emerging health problems of mountain gorillas.

Across the different systems investigated, parasite communities were shaped by interactions between host characteristics, parasite biology and ecological conditions. Host phylogeny and physiological compatibility influence which parasites can establish in particular hosts, while behaviour, habitat use and environmental conditions determine opportunities for exposure. Human activities can modify these relationships, but their effects are not uniform. Our studies showed both reductions in gastrointestinal symbiont diversity in disturbed habitats and changes in parasite occurrence or infection intensity associated with environmental and host-related factors. Similarly, the mountain gorilla studies demonstrated that even closely related host populations can experience different parasite-associated health problems. These findings underline the importance of investigating individual parasite taxa and their ecological context rather than assuming that parasite presence, environmental disturbance or increased infection intensity will necessarily have the same consequences across host populations.

Several questions remain unresolved. In particular, we still have limited knowledge of which hosts maintain the transmission of many gastrointestinal parasites, how frequently parasite lineages move between host species, and why infections that appear well tolerated in some populations are associated with severe disease in others. Addressing these questions is now a central aim of my research and guides the ongoing work of my research group. We are increasingly moving towards genome-wide approaches to investigate parasite population structure, evolutionary relationships and potential adaptations to different hosts. One particularly important question is whether parasite lineages originating from non-human primates can establish sustained transmission within human populations, or whether their persistence depends on repeated transmission from wildlife reservoirs. We have also started investigating parasite DNA in soil samples to identify potential hotspots of environmental contamination and better understand where opportunities for transmission arise in habitats shared by humans, wildlife and domestic animals. At the same time, we aim to better understand the host side of these relationships, including differences in immune responses that may help explain why strongylid infections are associated with severe disease in mountain gorillas but not with comparable clinical problems in western lowland gorillas.

Understanding why gastrointestinal parasites cause severe disease in some host populations is also important for developing better approaches to disease management. This is particularly relevant for mountain gorillas, where anthelmintic treatments are already used to manage gastrointestinal infections. Our ongoing research therefore extends beyond identifying the parasites associated with disease to exploring potential alternatives for their control. We are investigating plants traditionally used to treat parasitic infections in local human communities, together with plants consumed by wild primates, to identify potential natural anthelmintic compounds. We hope to contribute to more effective and

environmentally sustainable management of gastrointestinal parasitic diseases, particularly in endangered wildlife populations.

The broader conclusion of this habilitation is that gastrointestinal parasites need to be studied as part of dynamic systems involving multiple hosts and their shared environments. This is particularly important at the human–wildlife interface, where parasite circulation may have implications for human health, domestic animals and wildlife conservation. A One Health approach provides a useful framework for bringing these components together, but its success depends on understanding the biology of the parasites themselves. By combining increasingly detailed molecular identification with ecological and health-related information, we can move beyond describing parasite diversity towards a better understanding of the processes that determine parasite transmission and its consequences.

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8. List of publications included in the thesis

This habilitation thesis brings together 26 selected scientific publications organised into four thematic areas. The publications are listed according to the order in which they are discussed in the thesis, rather than chronologically. For clarity, they are grouped under the corresponding chapter headings.

Parasite diversity: from microscopy to metabarcoding and genomics

8.1 Bradbury, R.S., Pafčo, B., Nosková, E., Hasegawa, H., 2021. *Strongyloides* genotyping: a review of methods and application in public health and population genetics. Int. J. Parasitol. 51, 1153–1166. https://doi.org/10.1016/j.ijpara.2021.10.001

8.2. Šlapeta, J., Krücken, J., Rojas, A., Chambers, A., Melville, L.A., Martínez-Valladares, M., Canton, C., Francis, E.K., Zahid, O., Albuquerque, A.C.A., Bartley, D.J., Bassetto, C.C., Byrne, O., Colella, V., Costa-Junior, L.M., Doyle, S.R., Evans, M., Ghafar, A., Godoy, P., Hayashi, N., Helal, M.A., Huggins, L.G., Jabbar, A., Jones, R.A., Karani, B.E., Liron, J.P., Maté, L., McEvoy, A., Mohammedsalih, K.M., Mulcahy, G., Nielsen, M.K., Pafčo, B., Peachey, L.E., Robleto-Quesada, J., von Samson-Himmelstjerna, G., de Sousa-Paula, L.C., Gilleard, J.S., 2026. Ten simple rules for implementing deep amplicon sequencing in parasitology. Int. J. Parasitol. 56, 104751. https://doi.org/10.1016/j.ijpara.2025.11.003

8.3 Mason, B., Petrželková, K.J., Kreisinger, J., Böhm, T., Červená, B., Fairet, E., Fuh, T., Gomez, A., Knauf, S., Maloueki, U., Modrý, D., Shirley, M.H., Tagg, N., Wangue, N., Pafčo, B., 2022a. Gastrointestinal symbiont diversity in wild gorilla: a comparison of bacterial and strongylid communities across multiple localities. Mol. Ecol. 31, 4127–4145. https://doi.org/10.1111/mec.16558

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8.5 Pafčo, B., Nosková, E., Ilík, V., Anettová, L., Červená, B., Kreisinger, J., Pšenková, I., Václavek, P., Vyhlídalová, T., Ježková, J., Malát, K., Mihalca, A.D., Modrý, D., 2024. First insight into strongylid nematode diversity and anthelmintic treatment effectiveness in beef cattle in the Czech Republic explored by HTS metagenomics. Vet. Parasitol. Reg. Stud. Rep. 47, 100961. https://doi.org/10.1016/j.vprsr.2023.100961

8.6 Zhao, H., Haidamak, J., Nosková, E., Ilík, V., Pafčo, B., Ford, R., Masiria, G., Maure, T., Kotale, N., Pomat, W., Gordon, C., Navarro, S., Horwood, P.F., Constantinoiu, C., Greenhill, A.R., Bradbury, R.S., 2025. Insights into infant strongyloidiasis, Papua New Guinea. Emerg. Infect. Dis. 31, 1793–1801. https://doi.org/10.3201/eid3109.241923

8.7 Nosková, E., Svobodová, V., Hypská, V., Cerezo-Echevarria, A., Kurucová, T., Ilík, V., Modrý, D., Pafčo, B., 2024a. High-throughput sequencing of *Strongyloides stercoralis* – a fatal disseminated infection in a dog. Parasitology 151, 587–593. https://doi.org/10.1017/S0031182024000568

8.8 Ilík, V., Schwarz, E.M., Nosková, E., Pafčo, B., 2024. Hookworm genomics: dusk or dawn? Trends Parasitol. 40, 452–465. https://doi.org/10.1016/j.pt.2024.04.003

Host specificity, parasite circulation among host species and zoonotic potential

8.9 Pafčo, B., Kreisinger, J., Čížková, D., Pšenková-Profousová, I., Shutt-Phillips, K., Todd, A., Fuh, T., Petrželková, K.J., Modrý, D., 2019. Genetic diversity of primate strongylid nematodes: do sympatric nonhuman primates and humans share their strongylid worms? Mol. Ecol. 28, 4786–4797. https://doi.org/10.1111/mec.15257

8.10. Ilík, V., Kreisinger, J., Modrý, D., Schwarz, E.M., Tagg, N., Mbohli, D., Nkombou, I.C., Petrželková, K.J., Pafčo, B., 2023. High diversity and sharing of strongylid nematodes in humans and great apes co-habiting an unprotected area in Cameroon. PLoS Negl. Trop. Dis. 17, e0011499. https://doi.org/10.1371/journal.pntd.0011499

8.11 Vlčková, K., Kreisinger, J., Pafčo, B., Čížková, D., Tagg, N., Hehl, A.B., Modrý, D., 2018. Diversity of *Entamoeba* spp. in African great apes and humans: an insight from Illumina MiSeq high-throughput sequencing. Int. J. Parasitol. 48, 229–239. https://doi.org/10.1016/j.ijpara.2017.11.008

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8.14 Nosková, E., Ilík, V., Singa Niatou, F.S., Dumas, L., Fuh, T., Dicky, J.-F., Kurucová, T., Baláž, V., Petrželková, K.J., Pafčo, B., 2026. *Strongyloides* genetic diversity among humans, dogs, and nonhuman primates, Central African Republic, 2016–2022. Emerg. Infect. Dis. 32, 349–359. https://doi.org/10.3201/eid3203.250526

8.15 Bukamba, N., Sambucci, K.M., Nosková, E., Baláž, V., Okello, R.O., Muhumuza, D.D., Nizeyimana, F., Sargison, N.D., Kelly, R.F., Ssebide, B., Evans, T.S., Gilardi, K., Petrželková, K.J., Pafčo, B., Červená, B., 2026. The potential threat of *Strongyloides* spp. to mountain gorillas and public health in Bwindi Impenetrable National Park, Uganda. Parasitol. Res. 125, 52. https://doi.org/10.1007/s00436-026-08643-z

8.16 Upadhayay, P., Kváč, M., Sambucci, K.M., Nosková, E., Ilík, V., Pafčo, B., Holubová, N., Červená, B., Schoof, V.A.M., 2026. Overlap in parasite communities among vervet monkeys, humans, livestock and dogs at Lake Nabugabo, Uganda. Parasitology 153, 796–812. https://doi.org/10.1017/S0031182026101978

Ecological and anthropogenic drivers of parasite communities

8.17 Barelli, C., Pafčo, B., Manica, M., Rovero, F., Rosà, R., Modrý, D., Haufe, H.C., 2020. Loss of protozoan and metazoan intestinal symbiont biodiversity in wild primates living in unprotected forests. Sci. Rep. 10, 10917. https://doi.org/10.1038/s41598-020-67959-7

8.18 Mason, B., Piel, A.K., Modrý, D., Petrželková, K.J., Stewart, F.A., Pafčo, B., 2022b. Association of human disturbance and gastrointestinal parasite infection of yellow baboons in western Tanzania. PLoS ONE 17, e0262481. https://doi.org/10.1371/journal.pone.0262481

8.19 Shutt-Phillips, K., Pafčo, B., Heistermann, M., Kasim, A., Petrželková, K.J., Profousová-Pšenková, I., Modrý, D., Todd, A., Fuh, T., Dicky, J.F., Bopalanzognako, J.B., Setchell, J.M., 2021. Fecal glucocorticoids and gastrointestinal parasite infections in wild western lowland gorillas (*Gorilla gorilla gorilla*) involved in ecotourism. Gen. Comp. Endocrinol. 312, 113859. https://doi.org/10.1016/j.ygcen.2021.113859

8.20 Petrželková, K.J., Uwamahoro, C., Pafčo, B., Červená, B., Samaš, P., Mudakikwa, A., Muvunyi, R., Uwingeli, P., Gilardi, K., Nziza, J., Noheri, J.B., Eckardt, W., Ndagijimana, F., Ssebide, B., Okwirokello, R., Nizeyimana, F., Kambale Syaluha, E., Nzayisenga, G., Flores Girón, L., Bahizi, M., Ntwari, A.E., Lukusa, J.-P., Tumushime, J.C., Mangura, D., Mapilanga, J., Kalonji, A., Aruho, R., Stryková, A., Tehlarová, Z., Cameira, R., Lowenstine, L., Šlapeta, J., Romportl, D., Ferrari, N., Cranfield, M., Modrý, D., 2021. Heterogeneity in patterns of helminth infections across populations of mountain gorillas (*Gorilla beringei beringei*). Sci. Rep. 11, 10869. https://doi.org/10.1038/s41598-021-89283-4

8.21 Petrželková, K.J., Samaš, P., Romportl, D., Uwamahoro, C., Červená, B., Pafčo, B., Prokopová, T., Cameira, R., Granjon, A.C., Shapiro, A., Bahizi, M., Nziza, J., Noheri, J.B., Syaluha, E.K., Eckardt, W., Ndagijimana, F., Šlapeta, J., Modrý, D., Gilardi, K., Muvunyi, R., Uwingeli, P., Mudakikwa, A., Mapilanga, J., Kalonji, A., Hickey, J.R., Cranfield, M., 2022. Ecological drivers of helminth infection patterns in the Virunga Massif mountain gorilla population. Int. J. Parasitol. Parasites Wildl. 17, 174–184. https://doi.org/10.1016/j.ijppaw.2022.01.007

From infection to disease: health consequences of gastrointestinal parasites in wild primates

8.22 Červená, B., Prokopová, T., Cameira, R.M., Pafčo, B., Samaš, P., Romportl, D., Uwamahoro, C., Noheri, J.B., Ntwari, A.E., Bahizi, M., Nzayisenga, G., Nziza, J., Gilardi, K., Eckardt, W., Ndagijimana, F., Mudakikwa, A., Muvunyi, R., Uwingeli, P., Cranfield, M., Šlapeta, J.,

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8.1

Bradbury, R.S., Pafčo, B., Nosková, E., Hasegawa, H.

2021

***Strongyloides* genotyping: a review of methods and application in public health and population genetics.**

Int. J. Parasitol. 51, 1153–1166. https://doi.org/10.1016/j.ijpara.2021.10.001

International Journal for Parasitology 51 (2021) 1153–1166

Contents lists available at ScienceDirect

International Journal for Parasitology

journal homepage: www.elsevier.com/locate/ijpara

Invited Review

Strongyloides genotyping: a review of methods and application in public health and population genetics

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ARTICLE INFO

Article history:
Received 21 July 2021
Received in revised form 7 October 2021
Accepted 11 October 2021
Available online 30 October 2021

Keywords:
Strongyloides stercoralis
Strongyloides fuelleborni
Strongyloides
Strongyloidiasis
Genotyping
Genome
Epidemiology

ABSTRACT

Strongyloidiasis represents a major medical and veterinary helminthic disease. Human infection is caused by *Strongyloides stercoralis*, *Strongyloides fuelleborni fuelleborni* and *Strongyloides fuelleborni kellyi*, with *S. stercoralis* accounting for the majority of cases. *Strongyloides f. fuelleborni* likely represents a zoonosis acquired from non-human primates (NHPs), while no animal reservoir for *S. f. kellyi* infection has been found. Whether *S. stercoralis* represents a zoonosis acquired from dogs and cats remains unanswered. Over the past two decades various tools have been applied to genotype *Strongyloides* spp. The most commonly sequenced markers have been the hyper-variable regions I and IV of the 18S rRNA gene and selected portions of the cytochrome c oxidase subunit I gene. These markers have been sequenced and compared in *Strongyloides* from multiple hosts and geographical regions. More recently, a machine learning algorithm multi-locus sequence typing approach has been applied using these markers, while others have applied whole genome sequencing. Genotyping of *Strongyloides* from dogs, cats, NHPs and humans has identified that *S. stercoralis* likely originated in dogs and adapted to human hosts. It has also been demonstrated that *S. stercoralis* is distinct from *S. f. fuelleborni* and *S. f. kellyi*. Two distinct genetic clades of *S. stercoralis* exist, one restricted to dogs and another infecting humans, NHPs, dogs and cats. Genotyping of *S. f. fuelleborni* has identified two separate clades, one associated with African isolates and another Indochinese peninsular clade. This review summarises the history and development of genotyping tools for *Strongyloides* spp. It describes the findings of major studies to date in the context of the epidemiology and evolutionary biology of these helminths, with a specific focus on human-infecting species.

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Šlapeta, J., Krücken, J., Rojas, A., Chambers, A., Melville, L.A., Martínez-Valladares, M., Canton, C., Francis, E.K., Zahid, O., Albuquerque, A.C.A., Bartley, D.J., Bassetto, C.C., Byrne, O., Colella, V., Costa-Junior, L.M., Doyle, S.R., Evans, M., Ghafar, A., Godoy, P., Hayashi, N., Helal, M.A., Huggins, L.G., Jabbar, A., Jones, R.A., Karani, B.E., Liron, J.P., Maté, L., McEvoy, A., Mohammedsalih, K.M., Mulcahy, G., Nielsen, M.K., Pafčo, B., Peachey, L.E., Robleto-Quesada, J., von Samson-Himmelstjerna, G., de Sousa-Paula, L.C., Gilleard, J.S.

2026

Ten simple rules for implementing deep amplicon sequencing in parasitology.

Int. J. Parasitol. 56, 104751. https://doi.org/10.1016/j.ijpara.2025.11.003

International Journal for Parasitology 56 (2026) 104751

Contents lists available at ScienceDirect

International Journal for Parasitology

journal homepage: www.elsevier.com/locate/ijpara

Current Opinion

Ten simple rules for implementing deep amplicon sequencing in parasitology

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ARTICLE INFO

Article history:
Received 13 October 2025
Received in revised form 13 November 2025

ABSTRACT

Deep amplicon sequencing is transforming parasitology by enabling high-throughput profiling of parasite communities and detection of resistance-associated genetic variants. Despite its growing adoption, many researchers face challenges in implementation, and its full potential is often hindered by challenges in

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https://doi.org/10.1016/j.ijpara.2025.11.003
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Accepted 17 November 2025
Available online 21 November 2025

Keywords:
Guidelines
Microbiota
Metabarcoding
Amplicon profiling
Specimen handling
Methods
ASV
OTU

experimental design, including marker selection, data analysis and reproducibility. This article presents ten simple rules for applying deep amplicon sequencing in parasitology, developed through expert consensus at a deep amplicon sequencing symposium during the 2025 World Association for the Advancement of Veterinary Parasitology conference. These rules cover essential aspects from formulating research questions and choosing appropriate markers to managing data workflows and contributing to reference databases. We highlight the importance of integrating deep amplicon sequencing with traditional parasitological methods, ensuring transparent reporting and investing in capacity building. Whether you are new to deep amplicon sequencing or seeking to improve your current practices, these guidelines offer practical advice to enhance the robustness, reproducibility, and impact of your research. By adopting these principles, parasitologists can contribute to, and advance, a more reliable and collaborative scientific landscape.
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Introduction

The accessibility of PCR revolutionised parasitology in the late 1990s. Now, with the widespread availability of massively parallel sequencing, the field is undergoing a major transformation. Two commonly used applications are amplicon metabarcoding and targeted sequencing, of which both rely on deep sequencing of PCR amplicons. Practically speaking, amplicon metabarcoding used in parasitology aims to tell you which parasite species (e.g., based on ITS2 rRNA or *cox*1 gene sequences) are present in a complex or mixed parasite community within a sample such as faeces and can provide an estimate of their relative proportions (Avramenko et al., 2015; Pafčo et al., 2018). Targeted sequencing can reveal the proportion of those parasites that carry specific genetic variants, such as mutations in genes like acetylcholine receptor subunit (*acr*-8) or isotype-1 β-tubulin associated with levamisole and benzimidazole resistance, respectively (Avramenko et al., 2019; Francis et al., 2024). These techniques are increasingly applied to gastrointestinal parasites and haemoparasites; however, they are equally applicable to other parasite groups and systems (Talundzic et al., 2018; Flaherty et al., 2021; Titcomb et al., 2022; Antonopoulos et al., 2024; Šlapeta et al., 2024; Stensvold, 2024; Damnjanović et al., 2025; Peterson et al., 2025).

Amplicon metabarcoding profiles mixed communities, e.g., the 'nemabiome' (Avramenko et al., 2015), using universal or semi-universal primers targeting taxonomic genetic markers. The output is sequences or clusters of sequences, ASVs (amplicon sequence variants) or OTUs (operational taxonomic units), assigned to taxonomic ranks. If these clusters correspond to specific genes or regions of interest, they can also be used to detect genetic variants (e.g., SNPs), effectively serving as targeted sequencing. For example, in trichostrongyle nematodes of ruminants and hookworms of dogs, isotype-1 β-tubulin (*iso-1 β-tub*) amplicon sequencing has been used to survey community composition followed by quantifying resistance-associated allele frequencies (Avramenko et al., 2019; Venkatesan et al., 2023; Francis et al., 2024; Abdullah et al., 2025). In summary, targeted amplicon sequencing can serve multiple purposes, including metabarcoding (i.e., characterising parasite communities composed of multiple species) and detecting genetic variants in one or more genes, either within an individual organism (e.g., a single worm) or across a population of individuals from the same species (e.g., malaria parasites within a single infection). In this guide, we use the term deep amplicon sequencing broadly to encompass both community profiling and targeted sequencing applications.

Despite its potential, there are still significant barriers to the adoption and widespread use of deep amplicon sequencing in parasitology. These include limited access to sequencing platforms, the cost of reagents and a lack of bioinformatics expertise. Nevertheless, the use of these approaches continues to increase and expand. This momentum inspired the organisation of a deep amplicon sequencing symposium at the 2025 World Association for the Advancement of Veterinary Parasitology (WAAVP) conference in Curitiba, Brazil. The aim of the symposium was to identify key challenges in developing and adopting these methods and to provide a forum for researchers to share their experiences and develop high-level guidance for establishing good practices. While many of the examples that will be discussed stem from studies on nematode stages recovered from faecal samples from production animals, these serve as useful proxies for applying amplicon sequencing to other parasite groups, whether single-celled or multicellular and to sample types beyond faeces.

Here, we present ten simple rules for implementing deep amplicon sequencing to study parasites and parasite communities (Fig. 1). These rules are intended to help researchers make their work more robust, reproducible, and accessible, whether for peers, collaborators or their future selves. They support the thoughtful, rigorous application of molecular tools in parasitological investigations.

Rule 1: start with a clear question that is appropriate for deep amplicon sequencing that complements and enhances your parasitological investigations

The clarity of your scientific question determines the relevance and success of your deep amplicon sequencing approach. While deep amplicon sequencing is a powerful tool, it introduces uncertainty that can reduce comparability or lead to false positives through the misclassification of a target, or even false negative results when a target is not amplified despite being present (Alberdi et al., 2018). This uncertainty is partly due to the relative youth of deep amplicon sequencing methods compared to classical parasitological and established molecular techniques (e.g., qPCR) that have benefited from decades of standardisation and validation. Deep amplicon sequencing assays are not usually validated and so do not come with sensitivity and specificity values, unlike the majority of species-specific diagnostic assays, e.g., qPCR. These limitations can result in misleading conclusions.

Always begin by considering your study without deep amplicon sequencing: what would you learn using traditional parasitological or molecular methods alone without deep amplicon sequencing? Then ask how deep amplicon sequencing can enhance, not replace, those insights. For most applications in parasitology, deep amplicon sequencing should be viewed as a complementary technique, not a superior one. This approach is only as strong as the target sequence reference database, which depends on traditional parasitology skills for its development and maintenance (see Rule 9).

Rule 2: understand the nature of your samples and how this may affect your data and its interpretation

It is the researcher's responsibility to ensure that the sample is representative of the material being investigated, either through



Fig. 1. Ten simple rules for implementing deep amplicon sequencing in parasitology. Successful application of deep amplicon sequencing begins with a clearly defined research question. The central question should guide whether this approach enhances and is capable of addressing the biological or diagnostic challenge at hand. Surrounding this core, the rules aim to balance resolution, feasibility and reproducibility. Created in BioRender (https://BioRender.com/92os1dh).

sufficient parasite concentration or appropriate pre-processing. Deep amplicon sequencing relies on PCR, which can amplify even rare targets. This is particularly important for applications aimed at providing relative quantitation information as opposed to presence or absence. For multicellular eukaryotic parasites, a single organism (e.g., larva or egg) can produce a strong signal (Avramenko et al., 2015; Papaiakovou et al., 2019). Similarly, for single-cell parasites in clinical samples, targets may be present at a range of concentrations, and nested PCR protocols are often employed to amplify the signal (Talundzic et al., 2018; Cinar et al., 2020; Aranda-Diaz et al., 2025; Peterson et al., 2025; Rosado et al., 2025). PCR can also be subject to amplification bias, particularly if the target template is present at a low concentration, the quality of the DNA template is low, or if there is genetic variation in the primer binding site among species. Consequently, the signal may not always represent the parasite population in the sample or broader populations. To reduce ambiguity, duplicates (or even triplicates) can greatly reduce the incidence of amplification and sequencing errors in the resulting datasets. Deep amplicon sequencing produces point estimates shaped by multiple levels of subsampling, from host selection to DNA extraction and amplification. These estimates are further complicated by overdispersed distributions that are common in parasitology. Understanding and acknowledging these sources of uncertainty is essential for designing replicates and engaging meaningfully with a statistician.

One key factor is the number of individual parasites present in the sample that directly contributed to the template DNA preparation. Parasites can be enumerated directly via microscopy (e.g., in faeces or blood smears), or through qPCR, prior to further processing (Talundzic et al., 2018; Peterson et al., 2025). If parasites are harvested from faeces (e.g., eggs by flotation or larvae from coproculture) before DNA template is prepared, then the number of parasites contributing to the DNA preparation can be estimated by counting a representative aliquot (Doyle et al., 2019; Francis and Šlapeta, 2022). It should be noted that the faecal egg count is not always representative of the parasite burden in the host. If faecal DNA is being used as a template then the number of eggs can be estimated by extrapolating from faecal egg counts. For example, if there are 100 eggs per gram of faeces and DNA is extracted from 200 mg, only ~20 eggs may be present, assuming even distribution and theoretical perfect sample lysis efficiency. Hence, in this case, species proportions determined by deep sequencing or SNP frequency by amplicon sequencing are unlikely to be accurate. In the case of the third-stage larvae (L3) recovered from coprocultures, special attention must be paid to sample handling prior to incubation and incubation conditions to maximise development of all pos-

sible species present in the sample. The nature of your samples must be acknowledged, reported and you must consider the number of parasites in the sample as the starting point for DNA isolation, PCR and sequencing. Failure to do so risks inconclusive or erroneous results and post hoc corrections are rarely effective. Consider a pilot study that directly addresses the context of your material under your authentic field and laboratory constraints. Such a pilot study will ultimately strengthen your research.

Rule 3: choose a marker with the appropriate taxonomic resolution that answers your question

Select a genetic marker that provides the resolution needed for your research goals and target taxa. Contrary to popular belief, there is no universal marker that can barcode all life (Guo et al., 2022; Antil et al., 2023). Over the past 30 years, experts have independently tested and adopted different genetic markers for specific parasite groups. The literature and available sequence records offer a valuable opportunity to evaluate whether a marker suits your question before generating new data. It is your responsibility to understand what is commonly used in your field and what your peers will expect. If no suitable marker exists, designing a new assay and demonstrating the limitations of existing ones will be viewed positively by the community. Genomic data from verified parasite voucher material available through public genomic databases are invaluable in enabling design and redesign of deep amplicon sequencing assays. Today's accessibility of sequencing tools makes such contributions more feasible than ever.

If your question can be answered using an existing deep amplicon sequencing assay, consider using it rather than developing a new assay (Table 1). History has shown that no single marker works for all taxa or questions, so chasing a universal 'barcode' distracts from the real goal: answering your research question effectively. Focus on your research question first, not on the data you hope to generate. For example, the NC1–NC2 primers targeting the ITS2 rRNA gene region, central to the concept of the 'nemabiome' (Avramenko et al., 2015), are highly effective for many questions involving strongyle nematodes. Choosing a marker should be a strategic decision grounded in your study's objectives and host group, not a technical afterthought.

Understand the taxonomic limits of your chosen marker; some can resolve to genus, whereas others to species or even strain. For example, deep amplicon sequencing of the *acr*-8 exon 4 region, which includes the S168T-residue associated with levamisole resistance in *Haemonchus contortus* (Doyle et al., 2022), was found to be not variable enough to distinguish *H. contortus* from the closely related *H. placei* (Francis et al., 2024). This limitation was revealed through genome sequencing and highlights the importance of validating marker resolution before interpreting results. Comparative studies like these are essential to avoid overinterpreting deep sequencing data and to guide the development of more precise assays.

In other cases, marker limitations have led to the development of improved alternatives. For instance, the NC1–NC2 ITS2 assay performs well for many strongyle nematodes, but fails to distinguish certain cyathostomins in horses, such as *Coronocyclus coronatus* and *Cylicostephanus calicatus* (Bredtmann et al., 2019). A *cox*1-based assay was developed to resolve this ambiguity and even revealed potential cryptic species (Klass et al., 2025; Krücken et al., 2025). If your goal is to fully resolve cyathostomin species, *cox*1 will provide delineation of additional species relative to ITS2; however, if you are focused on detecting the major cyathostomins and large strongyle species like *Strongylus vulgaris*, the ITS2 assay remains effective. Knowing your marker's resolution helps you interpret results accurately and choose the right tool for the job. When multiple markers are applied, in general, interpret them within their inherent resolution limits. Avoid merging outputs from markers with different resolving power (e.g., ITS2 vs. *cox*1) into a single analysis without clearly stating that one provides genus-level information while the other resolves to species level. Misleading results can also arise from incorrectly linking genetic markers/alleles. When multiple sequence types are detected, determining which combinations originate from the same organism can be extremely challenging, or even impossible. Linking genetic markers should therefore be avoided.

Rule 4: design for resolution, not just amplification

Primer choice and amplicon length should be guided by the level of taxonomic resolution you need, not just PCR success. Historically, 300–600 bp amplicons were ideal for Sanger sequencing and became standard in multi-locus sequence typing (MLST) and early barcoding studies. With the rise of Illumina sequencing platforms, shorter fragments (<400 bp) became popular due to their compatibility with 2 × 250 or 2 × 300 bp sequencing kits and their ability to amplify degraded DNA. Newer technologies such as Oxford Nanopore Technologies (ONT) and Pacific Biosciences (PacBio) can sequence longer fragments, offering greater taxonomic resolution and flexibility in primer site selection (Baltrusis et al., 2022; Huggins et al., 2024a; Huggins et al., 2024b). An additional consideration for ONT-based deep sequencing is its higher error rate compared to short-read Illumina sequencing, which requires special attention during data analysis. Nonetheless, improved flow cell technology and numerous bioinformatic pipelines have been and are being developed to improve the error rate.

Chimeric reads, artificial sequences formed by the erroneous joining of distinct DNA templates during PCR, are a common artefact in deep amplicon sequencing, especially when high cycle numbers are used (Sze and Schloss, 2019). These chimeras can distort diversity estimates and lead to erroneous taxonomic assignments, yet they are often overlooked in sequencing workflows. To minimise their formation, PCR protocols should use the lowest effective number of cycles (at the exponential phase of amplification), high-fidelity polymerases and extended elongation times to reduce incomplete extensions that promote chimera formation (Sze and Schloss, 2019). On the bioinformatics side, chimera detection tools (e.g., UCHIME: https://drive5.com/, DADA2: https://benjjneb.github.io/dada2/, ChimeraSlayer: https://microbiomeutil.sourceforge.net/#A_CS) should be applied alongside a well-curated reference database to identify and remove artefactual sequences. Additionally, manual inspection of sequences with low classification confidence or unexpected taxonomic placements is essential, as residual chimeras can be mistakenly interpreted as novel variants or even new species. Implementing these strategies as standard practice is essential to ensure the accuracy and reliability of amplicon-based studies.

Designing new high-resolution assays is not trivial. For example, validating a *cox*1 assay for horse strongyles required sourcing morphologically verified voucher specimens and close collaboration between expert taxonomists and molecular biologists (Diekmann et al., 2025). This level of effort is rarely replicable without significant resources. While longer amplicons and novel markers may offer better resolution, they can only be used for accurate classification if suitable long-read sequencing data exists against which they can be matched (see Rule 9). Your assay design should balance resolution, feasibility and reproducibility, always guided by your research question.

Rule 5: anthelmintic resistance is a population-level phenomenon

Deep amplicon sequencing has been used to study anthelmintic resistance in two main ways. The first involves using ITS-2 'nema-

Table 1
Deep amplicon sequencing primers used in parasitology.

Primers		Gene, amplicon size	Taxonomical group	References
NC-1	ACGTCTGGTTCAGGGTTGTT	ITS-2, 300–500 bp	Strongylida	(Avramenko et al., 2015)
NC-2	TTAGTTTCTTTTCCTCCGCT			
Strongyl-ITS2-F	ACGTCTGGTTCAGGGTTG	ITS-2, 250–500 bp	Strongylida	(Pafčo et al., 2018)
Strongyl-ITS2-R	ATGCTTAAGTTCAGCGGGTA			
673f	ATYAGATACCGTCGTAGTCC	18S rDNA, 170 bp	*Entamoeba* spp.	(Vlčková et al., 2018)
942r	GTWCGGTCTTGGTAAGTTTTC			
ACB1_167_F	GGYGCAGGAAACAACTG	*iso-1 β-tub*, ~300 bp	Ancylostomatidae	(Jimenez Castro et al., 2019)
ACB1_167_R	CTTTGGTGAGGGGACAACA			
ACB1_200_F	GTRGTGGAGCCATACAATGC	*iso-1 β-tub*, ~300 bp	Ancylostomatidae	
ACB1_200_R	GGCATGAAGAAGTGAAGACGT			
Beta_Forward_Variant_1_Oo	ACGCACTCTTTGGGAGGAGG	*iso-1 β-tub*, ~300 bp	*Cooperia* spp., *Oesophagostomum* spp., *Haemonchus* spp., *Trichostrongylus* spp., *Teladorasgia* spp., *Ostertagia* spp.	(Avramenko et al., 2019)
Beta_Reverse_Variant_1_Oo	TGTGAGTTTTAGTGTGCGGAAG			
Beta_Forward_Variant_2_Co	TACGCATTCTCTTGGAGGAGG			
Beta_Reverse_Variant_2_Co	TGTGAGCTTCAATGTGCGGAA			
SBt_CoHc_F	CGCATTCWCTTGGAGGAGG			
SBt_CoHc_R	GTGAGYTTCAAWGTGCGGAAG			
SBt_TcTcol_F	CGCATTCYTTGGGAGGAGG			
SBt_TcTcol_R	GTGAGTTTYAAGGTGCGGAAG			
Beta_Forward_Nb_323bp	ACKCATTCWCTTGGAGGAGGC	*iso-1 β-tub*, ~300	*Nematodirus battus*	(Melville et al., 2020)
Beta_Reverse_Nb_323bp	GTSAGTTTCAATGTTCGGAAACAG			
Illumina_Beta_Exon3-4_Ospp_F_0	CCCGATAACTACGTGTTTGGA	*Iso-1 tub*, ~280 bp	*Oesophagostomum dentatum* *Oesophagostomum quadrispiculatum*	(Fischer et al., 2025)
Illumina_Beta_Exon3-4_Ospp_R	CTTGGGTGACGGTACIAC			
Illumina_Beta_Exon5-6_Ospp_F	GTCTCGGACACTGTGGTAGAGCCATACAAT	*Iso-1 tub*, ~230 bp	*Oesophagostomum dentatum* *Oesophagostomum quadrispiculatum*	
Illumina_Beta_Exon5-6_Ospp_F	AATTGACCAGGGAAGCGAAGGCAAGTAGTT			
Flsu_ex926F (S0925)	TAAACAGTGCGTGAAA	28S rDNA, ~200 bp	*Fasciola* spp.	(Calvani et al., 2020)
Flsu_ex806R (S0920)	CAAGCAGGTCCGTCAGTA			
NEW_HVR_I_F	GCTCATTATAACAGCTATAGACTACACGGTA	18S rDNA, HVR-I, ~434 bp	*Strongyloides* spp.	(Barratt et al., 2019)
NEW_HVR_I_R	CCACAACAATCATTTTATGCACTTGG			
NEW_HVR_IV_F	CGGGCCGGACACTATAAGG	18S rDNA, HVR-IV, ~255 bp		
NEW_HVR_IV_R	ATCTCTAAACAGGAACATAATGATCACTAC			
CM-V5F	GATTAGANACCNNNGTAGTTC	18S rDNA, ~200 bp	Microsporidia	(Trzebny et al., 2020)
CM-V5R	TAANCAGCACAMTCCACTC			
RLBF	GTGACCTATGAACTCAGGAGTC	18S rDNA, ~450 bp	Piroplasmida	(Ghafar et al., 2020)
RLBR	CTGAGACTTGCACATCGC			
LEV2 (S1128)	GCCGATGGTGAGCCTCATATTAC	*acr*-8, 180 bp	*Haemonchus* spp.	(Francis et al., 2024)
LEV6 (S1138)	GGWARCTGYCGCACATCTAAGAG			
Fil_COlint_F	TGATTGGTGGTTTTGGTAA	COI, ~650 bp	Filarioidea	(Huggins et al., 2024a)
Fil_COlint_R	ATAAGTACGAGTATCAATATC			
APICO_F_Mod	GCCAGTAGTCATATGCTTGTCT	18S rDNA, ~1,450 bp	Apicomplexa	(Huggins et al., 2024b)
Api_Illum_New-Rev	CTGTTATTGCCTYAAACTTCCTYG			
NGS_TFR1	TGCTTCAGTTCAGCGGGTCTTCC	ITS rDNA, ~300 bp	Trichomonadida	(Young et al., 2024)
NGS_TFR2	CGGTAGGTGAACCTGCCGTTGG			
EC1	GTGTGAGTCATCAGCTCACT	ITS-1/5.8S/ITS-2, 700–900 bp	Strongylida	(Charrier, 2024)
EC2	GATAGGTGGTCGACTTTCAC			
HaemNFI	CATATATTAAGAGAAITATGGAG	*cytb*, 478 bp (nested PCR)	avian *Plasmodium* and *Haemoproteus*	(Damnjanović et al., 2025)
HaemNR3	ATAGAAAGATAAGAAATACCATTC			
HaemF	ATGGTGCTTTCGATATATGCATG			
HaemR2	GCATTATCTGGATGTGATAATGGT			

biome' approach to estimate nematode species proportions before and after treatment, integrating this information into the faecal egg count reduction test (FECRT). This approach has been explored in recent studies (Queiroz et al., 2020; De Seram et al., 2023; Fischer et al., 2025; Leathwick et al., 2025) and is now referenced in the latest WAAVP guidelines for FECRT (Kaplan et al., 2023). However, this method is still in its infancy, and careful interpretation is essential (Krücken et al., 2024). It assumes that the post-treatment sampling represents a subset of the original (pre-treatment) population, making transparent sampling strategies and clear documentation of parasite numbers contributing to DNA extraction critical. In this regard, parasite populations under study should come from samples collected before and after treatment and ideally from the same animals, within a paired FECRT study, as recommended in the latest WAAVP guidelines (Kaplan et al., 2023). In contrast, in case of conducting an unpaired FECRT analyses, where a treated group is compared with a control group, this type of analysis may yield less robust results, since parasite populations can vary between individuals. Therefore, for a better interpretation of the results, transparent reporting of sampling strategy and input material is essential. If this approach is considered, clearly describe how samples were collected, concentrated and how many parasite stages (e.g., eggs or larvae) were included. Ideally, equal numbers of stages should be used in pre- and post-treatment samples; for example, if DNA is extracted from 2,000 larvae before treatment, aim for the same number after. If the treatment is effective, recovering a sufficiently high number of post-treatment stages (e.g., larvae) often requires culturing a significantly larger amount of faecal matter or simply may not be feasible. Crucially, avoid attempting to estimate relative abundances (=species proportions) when the number of recovered parasite stages falls below a determined minimum threshold, as this low input severely compromises the accuracy and precision of quantification. Replication improves reproducibility and rigor, even though it increases time and cost. Currently, integrating 'nemabiome' and FECRT data is an active area of research, and caution must be exer-

cised when presenting results due to existing caveats. Despite these challenges, the approach shows promise and is likely to become more robust with further research and validation.

The second application focuses on detecting SNPs (or other genetic variants) associated with anthelmintic resistance (Avramenko et al., 2019; Melville et al., 2020). When investigating resistance using deep amplicon sequencing, it is essential to focus on allele frequencies across parasite populations, not just the presence or absence of resistance markers. This is because faecal samples contain populations of eggs or larvae that reflect the genetic makeup of the parasite community within a host or group of hosts. For benzimidazole resistance, SNPs in the *iso-1 β-tub* gene are well-characterised in strongylid nematodes of ruminants and dogs, while levamisole resistance is associated with mutations in the *acr*-8 gene in ruminant nematodes (Avramenko et al., 2019; Venkatesan et al., 2023; Francis et al., 2024). However, the presence of a resistance allele does not automatically indicate clinical drug failure, and the latter can depend on the percentage of parasite stages which carry the alleles related to resistance and which genotypes are found. For levamisole, a S168T mutation frequency of >16 % in exon 4 of the *acr*-8 gene of *H. contortus* has been linked to drug failure, based on whole-genome sequencing of a single resistant isolate with >30 × coverage. Six independent deep amplicon sequencing assays applied to the same *H. contortus* sample showed allele frequencies ranging from 14 to 21 % (Francis et al., 2024).

Learning from experiences in medical parasitology, such as the application of deep amplicon sequencing to track antimalarial resistance, and in veterinary parasitology to monitor anthelmintic resistance, will ultimately guide how we deploy these tools (Aranda-Diaz et al., 2025; Guinda et al., 2025; Leathwick et al., 2025; Rosado et al., 2025). Given that drug resistance is a population-level phenomenon influenced by local selection pressures, researchers must exercise extreme caution when extrapolating resistance markers to new geographic regions or genetically distinct populations. In such contexts, drug failure may be driven by different alleles, alternative genes, or complex genetic backgrounds not assayed by the primary marker. Authors should report both the observed frequency and define two thresholds: one for assumed drug failure and one for the technical resolution of the assay to mitigate presence of false positives. These thresholds are inherently arbitrary and must be clearly justified and validated in the methods and contextualised in the discussion.

Rule 6: plan for data capture and analysis time

Bioinformatics takes time and expertise, budget for it in your project timeline and resources. For example, Illumina's MiSeq i100 Series can produce up to 100 million paired-end reads in a single run (Table 2). Even a small-scale deep sequencing study with 20 samples, each covered by 20,000 reads, involves processing hundreds of thousands of sequences. Projects involving dozens to hundreds of samples can be processed on personal computers; however, automation is essential to ensure data integrity, and larger projects will require access to a computing cluster (Sandve et al., 2013; Fierer et al., 2025). Manual adjustments should be minimised. This requires a tailored, reproducible workflow. The workflow must place special emphasis on automated quality control and filtering of low-quality reads; failure to rigorously filter reads can severely compromise the integrity of the downstream analysis, particularly in samples with poor sequencing results, often necessitating time-consuming manual intervention. Data analysis is conservatively as time-consuming as sample acquisition. If sample collection took six months, expect a similar timeframe for analysis by an equally experienced team member or collaborator. While collaboration is a good starting point, consider investing in personnel with computational skills or training interested lab members if deep amplicon sequencing becomes central to your research.

Inclusion and full analysis of adequate positive and negative controls is essential for reliable data interpretation. Contamination is a known issue in PCR workflows, but it becomes even more critical when handling large sample numbers in high-throughput sequencing. If you are analysing 300+ samples, a single negative control is insufficient. Another key consideration is determining minimum read depth thresholds, both total mapped reads per sample and per ASV. These thresholds depend on the nature of the experiment, sample type, contamination levels in controls and the research question. This becomes particularly complex in studies involving FECRTs (see Rule 5), where post-treatment samples may contain very few surviving parasites. In such cases, meeting general criteria for minimum egg or read counts may not be feasible. While no universal threshold can be recommended, it is essential to be transparent about these criteria when reporting and publishing your data.

One effective approach to improving the signal-to-noise ratio in deep amplicon sequencing studies is to include technical replicates and only retain ASVs or OTUs consistently detected across replicates. This helps distinguish true biological signals from stochastic amplification artefacts. Additionally, incorporating traceable synthetic DNA positive controls or verified true-positive samples allows for the establishment of read count thresholds, which can be used to assess background contamination and define criteria for parasite positivity (see Flaherty et al., 2018; Huggins et al., 2024b). Equally important is the inclusion of multiple true negative controls in each sequencing run. These controls help detect low-level background noise introduced by index cross talk, such as barcode hopping or index bleeding, common in multiplexed Illumina sequencing. Unlike laboratory contamination, which typically results in higher read counts, index cross talk introduces low-abundance artefactual reads that may appear in negative samples. Monitoring this background signal enables the rational definition of a minimum read threshold that distinguishes true positives from noise (Fierer et al., 2025). Together, these strategies provide a robust framework for improving the reliability of parasites detection and reducing false positives in high-throughput sequencing workflows.

Medium and large-scale projects require robust sample tracking systems that capture both sequence data and metadata. A Laboratory Information Management System (LIMS) can support this need by managing samples, workflows, and data efficiently. These systems are common in diagnostic labs and may be available through institutional partnerships as already implemented in global SARS-CoV-2 monitoring (Maier et al., 2021). However, they are not trivial to implement and represent a significant investment. Still, they offer long-term benefits in accuracy, reproducibility and scalability. Whether using a simple spreadsheet or a full LIMS, the goal remains the same: ensure that your data capture and analysis pipeline is well-planned, documented and aligned with the scale of your project.

Rule 7: make your data and analysis public

Share your raw data, metadata, and analysis pipeline to support reproducibility and advance the field. At a minimum, you should be able to reproduce your own results (Sandve et al., 2013). Even small deep amplicon sequencing projects generate tens to hundreds of thousands of sequences, requiring careful execution and documentation. Reproducibility depends on tracking every step of your workflow, from sample collection, storage and processing to bioinformatics. Record all relevant details, including software

Table 2
Sequencing platforms and their actual and potential capacity for performing deep amplicon sequencing.

Sequencing platforms and expected output			Multiplexing scenarios [**]		
Platform	Read length (max)	Single read count (max expected) [*]	Potential number of samples with 20,000 reads per amplicon [***]	96-plex: reads per amplicon per sample [****]	384-plex: reads per amplicon per sample [****]
Illumina iSeq 100	2 × 150 bp	4,000,000	200	41,667	10,417
Illumina MiSeq	2 × 300 bp	25,000,000	1250	260,417	65,104
Illumina MiSeq i100	2 × 300 bp	100,000,000	5,000	1,041,667	260,417
Illumina NextSeq 550	2 × 150 bp	400,000,000	20,000	4,166,667	1,041,667
Illumina NextSeq 1000	2 × 300 bp	400,000,000	20,000	4,166,667	1,041,667
ONT Flongle	500 bp to 5 kb (much longer possible)	2,800,000	140	29,167	7,292
ONT MinION	500 bp to 5 kb (much longer possible)	20,000,000	1,000	208,333	52,083
ONT PromethION	500 bp to 5 kb (much longer possible)	290,000,000	14,500	3,020,833	755,208
PacBio Revio	up to 15–20 kb	20,000,000	1,000	208,333	52,083
PacBio Vega	up to 15–20 kb	8,000,000	400	83,333	20,833
PacBio Onso	2 × 150 bp	1,000,000,000	50,000	10,416,667	2,604,167
Element Biosciences AVITI LT	2 × 300 bp	500,000,000	25,000	5,208,333	1,302,083
Element Biosciences AVITI	2 × 300 bp	1,000,000,000	50,000	10,416,667	2,604,167
MGI DNBSEQ-G99	2 × 300 bp	100,000,000	5,000	1,041,667	260,417

Note: Sequencing technologies evolve rapidly. For the most current options and guidance, consult your institutional core facility or local sequencing provider to identify the most suitable platform for your deep amplicon sequencing needs.

[*] ONT flow cells generate an approximate number of bases sequenced, depending on the flow cell and run time, rather than a defined number of reads. Here, read stats are based on the total potential output divided by an average amplicon length of 1,000 bp.

[**] Multiplexing scenarios are used to illustrate the number of samples that could be processed, and the potential outputs generated. All assume the use of a single amplicon per sample, such as used in the "nemabiome" assays. However, combining multiple amplicons are possible in a single reaction, which will require recalculation of the multiplexing and read/sample calculation

[***] 20,000 reads/amplicon is an arbitrary number, and may be higher or lower depending on the need.

[****] Experimental designs based on 96- or 384-plex of samples are commonly used. Here, the number of reads are shown; the choice of platform will influence the read depth per amplicon, which may need to be higher or lower, depending on the need.

names, versions, parameters and input files. Whenever possible, automate your workflow to avoid manual steps like copy-pasting, which are error-prone and hard to trace. If manual operations are unavoidable, document them clearly, noting which files were modified and why. Detailed notes from both wet lab and dry (=computational) lab experiments are the foundation of good science.

A robust deep sequencing publication includes three key ingredients. First, deposit raw sequence data, including control samples, in a permanent public repository such as the NIH Sequence Read Archive (SRA; https://www.ncbi.nlm.nih.gov/sra). Controls help assess signal-to-noise ratios and validate your findings (Reitmeier et al., 2021; Fierer et al., 2025). Second, provide rich metadata in supplementary information of the publication and with the submission of raw data to repositories. What may seem trivial to you could be essential for someone conducting a *meta*-analysis. Ideally, metadata should be linked directly to sequence identifiers. Third, share your analysis pipeline alongside your publication. Most journals now support or require data accessibility statements with links to repositories for data and code such as GitHub (https://github.com/), Zenodo (https://zenodo.org/), FigShare (https://figshare.com/), LabArchives (https://www.labarchives.com/) or similar. Making your work reproducible sends a strong message of rigor, trustworthiness, and transparency.

Rule 8: invest in implementation from sample processing to analysis

The time and effort you put into learning and refining your laboratory workflow directly impacts the quality and credibility of your results. While deep amplicon sequencing can become a routine tool in parasitological investigations, deploying it well requires foundational knowledge in molecular biology, particularly PCR and sequence analysis. This includes *in silico* primer evaluation or design, understanding DNA extraction and recognising the limitations of PCR. *In silico* work for parasites is now well supported by several user-friendly tools available for desktop computers (e.g., https://www.arb-silva.de/, https://pr2-database.org/, https://www.nemabiome.ca/, https://eukaryome.org/) (Guillou et al., 2013; Glockner et al., 2017; Workentine et al., 2020; Tedersoo et al., 2024). For example, PrimerTC (Primer Taxonomic Conservation) is a tool assessing the phylogenetic conservation of primers targeting different regions of the rRNA cistron in nematodes to allow more tailored 'nemabiome' studies (Charrier et al., 2024; https://www.nemabiome.ca/primertc). Alternatively, OBITools is a versatile tool specifically developed for analysing deep amplicon sequencing data in a DNA metabarcoding context, taking into account taxonomic information including primer design (see a hands on OBITools tutorial implemented in Galaxy: https://gxy.io/GTN:T00124) (Boyer et al., 2016). Contamination during sampling and laboratory work is inevitable in molecular workflows, so users must be trained in basic mitigation strategies and contamination-aware set up and design, is integral to rigorous implementation of deep amplicon sequencing (e.g., Reitmeier et al., 2021). If your lab lacks experience in PCR-based methods, build capacity first through collaboration, training, or mentorship before implementing deep amplicon sequencing. Another possible option, particularly for parasitology laboratories with a more clinical focus, is to perform sampling, DNA extraction and PCR amplification in-house, while subcontracting or collaborating for the sequencing and bioinformatic analysis. However, for these options, it is essential to clearly understand Rules 3, 4 and 6, which must be carefully planned and explained in detail to the subcontracted facility.

Though the per-sample deep sequencing cost may seem low (e.g., ~US$20 in August 2025), even a moderately sized project (e.g., 400 samples) can require significant investment in consumables alone. Even small projects can become overwhelming without careful planning. Always test and troubleshoot your pipeline on a subsample of, for example, known positive control or a mock community. Be sceptical of your results and consider alternative explanations. Always examine and check your data and results in the biological context of the experiment to help identify flags for potential errors and problems. Amplicon sequencing rarely yields even read coverage across samples, some may have a few thousand reads, others hundreds of thousands. Ensuring a minimum number of reads, and a minimum number (or proportion) of hits to a target, is important. Similarly, depending on the research question, samples with excessive numbers of reads can be subsampled to 10,000 or 20,000 reads per sample to improve processing efficiency without compromising results (see also Hong et al., 2022; Schloss, 2024). Subsampling (or rarefying) reads needs to be approached with caution in studies where low-abundance taxa (e.g., <1/10,000 reads in at least 10 % of samples) may be inconsistently detected across repeated subsamples.

Ultimately, the investment in implementation, in particular when paired with traditional parasitological methods, can elevate your research. It will not only strengthen your findings but also provide rewarding opportunities for your students, collaborators, and institution.

Rule 9: your results are only as good as the reference database

Deep amplicon sequencing relies on a reference database of known sequences from defined species to map or compare your sequencing reads. Database completeness and curation are critical to the success of the approach, and so knowing your sequence database and its limitations is important for the interpretation of your results. Most available marker gene sequences are heavily skewed toward parasites of humans, production animals such as sheep and cattle, with some additional coverage for horse nematodes and companion animals. For many other hosts, baseline data on parasite diversity and within-species variation is still in its infancy. For example, soil arthropod fauna remains insufficiently documented, with most species unrepresented in DNA barcode libraries (Recuero et al., 2023). Even for well-studied systems, sequence data often reflects the local context of contributors, typically from high-income countries. If you are working in different climatic or geographic regions, especially in low- and middle-income countries, your animals may host locally adapted or uncharacterised parasites (Papaiakovou et al., 2025). These may differ genetically from reference sequences, even if they belong to the same species. If your reference dataset is in anyway unique, publish your database alongside your publication as supplementary data or in a repository (see Rule 7).

Recognising the local parasite context and verifying the identity of your target organisms is essential. Before sequencing, compare your expected taxa against available databases using in silico tools. A comprehensive and regularly updated set of nematode rRNA gene databases (ITS1, ITS2, ITS1/5.8S/ITS2, 18S and 28S) are available at https://www.nemabiome.ca/ (Workentine et al., 2020; Charrier et al., 2024). It is a critical best practice to prioritise curated reference resources over raw repositories (e.g., GenBank; https://www.ncbi.nlm.nih.gov/genbank/) whenever possible, as uncurated public databases are known to harbor errors in taxonomic assignment that can compromise the resulting diversity profile. If key species are missing or underrepresented, plan accordingly (see Rule 10). Dealing with unidentified ASVs or OTUs post hoc can delay interpretation and reduce confidence in your results. Missing expected species in your output may reflect database gaps rather than true absence. Similarly, looking for unusual mapping or additional diversity in your data might reveal cryptic species/sequences that could be added to the database. Obtaining verified voucher specimens and expert morphological confirmation is time-consuming and not always feasible. Integrating database awareness into your study design will help avoid misinterpretation and strengthen the reliability of your findings. Sequencing information in many areas of parasitology is constantly growing and missing species are being added, so that re-analysis of sequence data at a later stage may allow to add new insight.

Rule 10: fill the gaps in the database

If your species or marker is not represented in public databases, generate reference sequences using verified voucher specimens and publish about them. Relying on second-hand data can lead to misidentification, especially if the original sequence lacks proper documentation. If a parasite species is central to your study, generating your own reference sequence from locally relevant, morphologically verified material will strengthen your work. Making your genomic materials and data available through a public repository, such as by registering them via the Global Genome Biodiversity Network (GGBN; https://www.ggbn.org/), offers an opportunity to future-proof your contribution to the global effort to map biodiversity. When using public databases like GenBank, critically assess the authenticity of the sequences, check for associated publications and confirm that the sequence is linked to a voucher specimen, ideally deposited in a museum or verified by a taxonomic expert. Use phylogenetic tree reconstruction as a primary quality assurance step to confirm the taxonomic placement of novel ASVs/OTUs or sequences of questionable quality. Aligning sequences with robustly classified references allows for visual corroboration of identity and can help detect cases of misclassification or potential cryptic species. Each reference sequence should be traceable to a reliable source.

One of the major challenges in parasitology is bridging the gap between historical morphological records and modern molecular data. The massively parallel sequencing of voucher specimens that are deposited in parasite museums offers an affordable and scalable solution (e.g., Deak and Šlapeta, 2025). For relatively little cost (~US $100 in August 2025), genome skimming can yield high-coverage data for multicopy markers such as rRNA genes (ITS1, ITS2) and mitochondrial genes (e.g., *cox*1), which can be assembled de novo and reanalysed as tools evolve. These deep sequencing datasets, when deposited as raw reads and linked to voucher material, are superior to single consensus Sanger sequences, because they preserve within-specimen variation and support future assay development. Generating genomic data for new or poorly described parasite species should no longer be aspirational (Šlapeta, 2013). These organisms deserve our investment so they can be included in current deep sequencing surveys and remain accessible for future research.

Conclusions

Deep amplicon sequencing has already transformed our understanding of parasite communities across hosts and facilitated the detection of drug resistance in diverse taxa. We believe this marks the beginning of a broader transformation in parasitology. With widespread adoption and a clear understanding of its strengths and limitations, deep amplicon sequencing will help define what infections are "normal" in ecosystems and elucidate the factors shaping parasite community composition and transmission across hosts (Deiner et al., 2017; Wood et al., 2025).

When coupled with well-curated reference databases, deep amplicon sequencing democratises species identification. It is independent of parasite life stage and relies solely on our ability to extract DNA. Applied to parasite communities, it enables the detection of species that are morphologically almost indistinguishable and would otherwise require highly trained expert taxonomists. However, deep amplicon sequencing does not replace taxonomy. Parasite taxonomy and nomenclature depend on expert taxonomists, integrating disparate data and using judgment, to distinguish biological signal from noise (Poulin and Presswell, 2022; Poulin et al., 2025).

We hope these ten simple rules will empower parasitologists to invest in capacity building, define clear research objectives and contribute to the development of databases tailored to specific parasite taxa, host species and ecosystems. By integrating deep sequencing with classical parasitology and embracing open data practices, we can build a more collaborative, reproducible and impactful future for the discipline.

CRediT authorship contribution statement

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Acknowledgements

This manuscript emerged from a symposium around presentations by JK, AR, AC, LAM, MM, CC, EKF and OZ and deep amplicon sequencing roundtable during the 30th Conference of the World Association for the Advancement of Veterinary Parasitology in August 2025 in Curitiba, Brazil organised by JŠ and JSG.

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2022a

Gastrointestinal symbiont diversity in wild gorilla: a comparison of bacterial and strongylid communities across multiple localities.

Mol. Ecol. 31, 4127–4145. https://doi.org/10.1111/mec.16558

Check for updates

Received: 28 December 2021 | Revised: 17 April 2022 | Accepted: 5 May 2022

DOI: 10.1111/mec.16558

ORIGINAL ARTICLE

MOLECULAR ECOLOGY WILEY

Gastrointestinal symbiont diversity in wild gorilla: A comparison of bacterial and strongylid communities across multiple localities

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Funding information
Grantová Agentura České Republiky, Grant/Award Number: 18-24345S and 22-16475S

Handling Editor: Jacob A Russell

Abstract

Western lowland gorillas (*Gorilla gorilla gorilla*) are Critically Endangered and show continued population decline. Consequently, pressure is mounting to better understand their conservation threats and ecology. Gastrointestinal symbionts, such as bacterial and eukaryotic communities, are believed to play vital roles in the physiological landscape of the host. Gorillas host a broad spectrum of eucaryotes, so called parasites, with strongylid nematodes being particularly prevalent. While these communities are partially consistent, they are also shaped by various ecological factors, such as diet or habitat type. To investigate gastrointestinal symbionts of wild western lowland gorillas, we analysed 215 faecal samples from individuals in five distinct localities across the Congo Basin, using high-throughput sequencing techniques. We describe the gut bacterial microbiome and genetic diversity of strongylid communities, including strain-level identification of amplicon sequence variants (ASVs). We identified strongylid ASVs from eight genera and bacterial ASVs from 20 phyla. We compared

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these communities across localities, with reference to varying environmental factors among populations, finding differences in alpha diversity and community compositions of both gastrointestinal components. Moreover, we also investigated covariation between strongylid nematodes and the bacterial microbiome, finding correlations between strongylid taxa and Prevotellaceae and Rikenellaceae ASVs that were consistent across multiple localities. Our research highlights the complexity of the bacterial microbiome and strongylid communities in several gorilla populations and emphasizes potential interactions between these two symbiont communities. This study provides a framework for ongoing research into strongylid nematode diversity, and their interactions with the bacterial microbiome, among great apes.

KEYWORDS
bacterial microbiome, gorilla, helminth, non-human primates, Strongylida

1365294x, 2022, 15, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/mec.16558 by Institute of Vertebrate

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Mason, B., Červená, B., Frias, L., Goossens, B., Hasegawa, H., Keuk, K., Langgeng, A., Majewski, K., Matsumoto, T., Matsuura, K., Mendonça, R., Okamoto, M., Peter, S., Petrželková, K.J., Sipangkui, S., Xu, Z., Pafčo, B., MacIntosh, A.J.J.

2024

Novel insight into the genetic diversity of strongylid nematodes infecting South-East and East Asian primates.

Parasitology 151, 514–522. https://doi.org/10.1017/S0031182024000386

Parasitology

cambridge.org/par

Novel insight into the genetic diversity of strongylid nematodes infecting South-East and East Asian primates

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Research Article

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Cite this article: Mason B *et al* (2024). Novel insight into the genetic diversity of strongylid nematodes infecting South-East and East Asian primates. *Parasitology* **151**, 514–522. https://doi.org/10.1017/S0031182024000386

Received: 12 January 2024
Revised: 21 March 2024
Accepted: 21 March 2024
First published online: 17 April 2024

Keywords:
helminth; metabarcoding; non-human primate; orangutan; strongylida

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Abstract

With many non-human primates (NHPs) showing continued population decline, there is an ongoing need to better understand their ecology and conservation threats. One such threat is the risk of disease, with various bacterial, viral and parasitic infections previously reported to have damaging consequences for NHP hosts. Strongylid nematodes are one of the most commonly reported parasitic infections in NHPs. Current knowledge of NHP strongylid infections is restricted by their typical occurrence as mixed infections of multiple genera, which are indistinguishable through traditional microscopic approaches. Here, modern metagenomics approaches were applied for insight into the genetic diversity of strongylid infections in South-East and East Asian NHPs. We hypothesized that strongylid nematodes occur in mixed communities of multiple taxa, dominated by *Oesophagostomum*, matching previous findings using single-specimen genetics. Utilizing the Illumina MiSeq platform, ITS-2 strongylid metabarcoding was applied to 90 samples from various wild NHPs occurring in Malaysian Borneo and Japan. A clear dominance of *Oesophagostomum aculeatum* was found, with almost all sequences assigned to this species. This study suggests that strongylid communities of Asian NHPs may be less species-rich than those in African NHPs, where multi-genera communities are reported. Such knowledge contributes baseline data, assisting with ongoing monitoring of health threats to NHPs.

Introduction

Non-human primates (NHPs) are among the most endangered of mammalian taxa (Estrada *et al*., 2017; Fernández *et al*., 2022). Asian NHPs are particularly threatened, with one of the largest proportions of threatened NHP species reported across global regions, second only to Madagascar (Fernández *et al*., 2022). Asian NHPs commonly feature among the International Union for Conservation of Nature's 'World's 25 Most Endangered Primates' and face a variety of conservation challenges (Mittermeier *et al*., 2006). Consequently, effective conservation strategies rely on understanding both their ecology and conservation threats. One such threat is the risk of disease, with various bacterial, viral and parasitic infections previously reported to have damaging consequences for NHP hosts (Chapman *et al*., 2005; Mul *et al*., 2007; Gillespie *et al*., 2008; Bicca-Marques *et al*., 2022). In particular, host–parasite interactions have long been associated with population dynamics of wildlife hosts (Hudson and Dobson, 1995). Wild NHPs host numerous parasites (Nunn *et al*., 2003; Frias and MacIntosh, 2020), including strongylid nematodes, a major radiation of parasitic helminths, herein referred to as strongylids. Strongylids are amongst the most common gastrointestinal parasites of wild NHPs, typically occurring as asymptomatic infections within such hosts (Cantacessi *et al*., 2012; Pafčo *et al*., 2018). However, clinical manifestations have been previously reported in both captive and wild NHPs (Pit *et al*., 2001; Mul *et al*., 2007; Muhangi *et al*.,

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2021). Strongylids are expected to occur in complex communities of multiple genera within NHP hosts, as is conventional in large terrestrial herbivores (Pafčo *et al.*, 2018).

While strongylids have been widely studied in both human medicine and veterinary sciences (Zajac, 2006; Cantacessi *et al.*, 2012), knowledge of these infections in wildlife is still limited (Krief *et al.*, 2008; Mclean *et al.*, 2012; Walker and Morgan, 2014). It is restricted by the indistinguishability of different species, or even genera, through traditional coproscopic approaches (Jex *et al.*, 2011; Pafčo *et al.*, 2018). Previously applied molecular approaches are time consuming and require either single larval specimens or species-specific primers. Such methodologies are insufficient for capturing the true genetic diversity of these complex communities, at risk of overlooking rarer taxa (Pafčo *et al.*, 2018). Technological advancements in high-throughput sequencing (HTS) now facilitate simultaneous sequencing of mixed DNA from entire communities, including identification of rare taxa (von Bubnoff, 2008), in a timely and cost-effective manner. In recent research, HTS methodologies have confirmed the presence of complex strongylid communities in various African NHPs (Pafčo *et al.*, 2019; Mason *et al.*, 2022). However, application is not currently widespread and these methods are yet to be applied to strongylid communities of Asian NHPs.

Recent research has revealed striking differences in strongylid communities of African great apes (Pafčo *et al.*, 2019; Ilík *et al.*, 2023), raising questions as to the drivers of these complex communities. Parasite phylogenies are sometimes thought to emulate the host phylogenies within which they evolved, as predicted by the host-parasite co-speciation hypothesis (Brooks, 1979). However, environmental parameters, such as temperature, humidity or vegetation, are known to influence longevity and transmission of parasite populations (Silangwa and Todd, 1964; Callinan and Westcott, 1986; Jex *et al.*, 2011). As soil-transmitted helminths, strongylids may be particularly influenced by environmental variation, due to development of infectious larvae involving free-living larval stages within the external environment (Kalousová *et al.*, 2014; Knapp-Lawitzke *et al.*, 2014). Similarly, parasite populations are also influenced by the presence or absence of sympatric species, be that other wildlife or domestic animals (Hatcher *et al.*, 2012). Existence of multiple potential hosts within a given area facilitates parasite sharing between host species, particularly among phylogenetically related hosts (Dallas *et al.*, 2019), including NHPs (Cooper *et al.*, 2012; Pafčo *et al.*, 2019). With a direct lifecycle, strongylids rely on overlapping host ranges for transmission (Pafčo *et al.*, 2019), without an intermediate or transport host to facilitate wider transmission.

Coproscopic studies have confirmed a high prevalence of strongylids in both South-East and East Asian primates (Arizono *et al.*, 2012; Frias *et al.*, 2021). To date, genetic research of strongylids in Asian NHPs has typically focused on *Oesophagostomum* spp., the so-called 'nodular worms'. Only one species of nodular worm, *O. aculeatum*, has been genetically identified in Asian NHPs thus far (Arizono *et al.*, 2012; Frias *et al.*, 2019; Yalcindag *et al.*, 2021). There are few reports of other strongylid taxa in Asian NHPs. For example, a previous molecular characterization identified the presence of a single *Ternidens deminutus* larva in a sampled community of Bornean NHPs (Frias *et al.*, 2019). However, due to this previous research using single larval specimens and relying on taxa-specific primers, the strongylid communities identified cannot be deemed conclusive. To build upon current knowledge, a holistic approach better able to capture the entire strongylid community, including rare taxa, is needed.

Here, a previously optimized HTS approach (Pafčo *et al.*, 2018) is utilized to shed light on the genetic diversity of strongylid communities infecting South-East and East Asian NHPs. This approach is applied to 5 distinct NHP populations, including a total of 6 primate species. In describing the strongylid communities of Bornean orangutans (*Pongo pygmaeus*), one of the only Asian great apes, and several sympatric NHP species, the aim is to provide a unique insight into the host–parasite co-speciation hypothesis within great apes. Insight into within species variation of NHP strongylid communities is also provided, through comparison of Japanese macaques (*Macaca fuscata)* from 3 isolated localities. We hypothesize that strongylid nematodes occur in mixed communities of multiple taxa, dominated by *Oesophagostomum*, matching previous findings based on single-specimen genetics.

Material and methods

Sample collection

In this study, 90 individual faecal samples were utilized, non-invasively collected from 6 different wild NHP species in South-East and East Asia, precisely across 5 localities in Malaysian Borneo and Japan. In Borneo, sampling was conducted at 2 localities: the Lower Kinabatangan Wildlife Sanctuary (LKWS) and Danum Valley Conservation Area (DVCA), both of which occur in the state of Sabah. At LKWS, 36 samples representing 5 primate species were collected: crab-eating macaque (*Macaca fascicularis*, $n = 17$), southern pig-tailed macaque (*Macaca nemestrina*, $n = 2$), proboscis monkey (*Nasalis larvatus*, $n = 4$), silvery lutung (*Trachypithecus* cristatus, $n = 5$), and Bornean orangutan (*Pongo pygmaeus*, $n = 2$). Except for Bornean orangutans, which were either followed or sampled opportunistically during their visits to the Centre, LKWS samples were collected during systematic boat surveys along the Kinabatangan River, with NHP species being opportunistically sampled when encountered at sleeping sites early in the morning. Visual appearance of faeces based on softness and presence of invertebrates was used to ensure that only fresh samples were collected. Finally, 12 identified Bornean orangutans inhabiting DVCA were sampled during standard focal follows of habituated individuals. Further sampling was conducted in Japan, with 48 samples collected from the 2 subspecies of Japanese macaques (*Macaca fuscata fuscata* and *M. f. yakui*) inhabiting 3 localities: Jigokudani ($n = 15$), Koshima ($n = 21$) and Yakushima ($n = 12$). At these 3 localities, the primates are habituated to human presence and each sample can be attributed to a known individual. Samples from all localities were fixed in ethanol as soon as possible after collection, always on the same day, with fixed samples then stored at environmental temperature before transportation to Wildlife Research Center, Kyoto University, Japan.

DNA isolation and sequencing

Total genomic DNA was extracted from faecal samples using a QIAamp DNA stool mini kit (Qiagen, Japan) following the manufacturer's recommendations. To ensure correct host identification of samples originating from unidentified hosts in LKWS, cytochrome b (*cytb*) gene fragments were amplified using the primer pair L14724/H15915 (CGAAGCTTGATATGAAAAACCATCGTTG/AACTGCAGTCATCTCCGGTTTACAAGAC – respectively) (Irwin *et al.*, 1991). Amplicons were then purified using the Agencourt AMPure system (Agencourt Bioscience Corp., USA) before sequencing in an ABI 3730xl DNA Analyzer (Applied Biosystems, USA). Resulting sequences were compared with GenBank template sequences to identify the correct primate species, described in full in Frias *et al.* (2018).

Strongylid presence was assessed in each sample using HTS, where the entire strongylid community was determined by

amplification of the second internal transcribed spacer (ITS-2) of nuclear DNA through PCR (polymerase chain reaction) using the forward primer Strongyl_ITS-2_F (ACGTCTGGTTCAGGGTTG) and the reverse Strongyl_ITS-2_R (ATGCTTAAGTTCAGCGGGTA) (Pafčo *et al.*, 2018). To generate HTS sequencing libraries a 2-step PCR approach that employs Nextera primer design was used (Pafčo *et al.*, 2018), with each sample tagged with a unique primer barcode, following Illumina libraries guide. Through the Illumina MiSeq platform the final library was sequenced using MiSeq Reagent Kit version 2 (2 × 250-bp pair-end reads, 500 cycles).

Bioinformatics and data analysis

Gene-specific primers were trimmed using SKEWER (Jiang *et al.*, 2014). Using DADA2, paired end reads were assembled (Callahan *et al.*, 2016). The dataset was then denoised, low-quality reads (expected error rate >1) eliminated and inflation of strongylid diversity by PCR/sequencing artefacts (chimeras) avoided by corrections in DADA2 (Callahan *et al.*, 2016). To taxonomically assign amplicon sequence variants (ASVs) the naïve Bayesian RDP classifier (Wang *et al.*, 2007) was employed in the DADA2 pipeline. With strongylid ITS-2 sequences, downloaded from the NCBI nr/nt database (200 top BLASTN hits for each ASVs), a training database of reference sequences for assignment was constructed. The dataset was then filtered to remove all unclassified ASVs, those not assigned to Strongylida. Phylogenetic maximum likelihood analysis was performed using the web version of IQ TREE (Trifinopoulos *et al.*, 2016), with integrated ModelFinder selecting the most suitable model based on Bayesian information criterion (BIC) (Kalyaanamoorthy *et al.*, 2017), after removal of outlying ASV sequences with both low abundance and prevalence and addition of *Oesophagostomum* spp. sequences available in the GenBank. The topology of phylogenetic trees was tested using 1000 replicates of ultrafast bootstrap (Hoang *et al.*, 2018) and Shimodaira–Hasegawa approximate likelihood ratio test (SH-aLRT) (Anisimova *et al.*, 2011). Through the model outputs, attention was also paid to pairwise distances between taxa, which were calculated from the alignments guided by Clustal Omega integrated into Geneious Prime 2023 2.1 (http://www.geneious.com). The trees were visualized and annotated in iTOL v5 (Letunic and Bork, 2021).

Differences in alpha diversity of strongylid communities were investigated by using the number of ASVs per sample as a proxy measure. Using the lme4 R package (de Boeck *et al.*, 2011; Bates *et al.*, 2015), a GLMM (generalized linear mixed model) with negative binomial distribution was applied, accounting for aggregated parasite distributions, to assess the effect of host species on alpha diversity, including study site as a random factor. To assess overdispersion and ensure correct model selection, residual diagnostics were implemented and visually inspected through the DHARMa R package (Hartig, 2020), with no significant deviation found. Then the drop1() function (again in lme4 R package) was implemented to determine the importance of host species as a model effector, with strong significance identified comparative to a null hypothesis. *Post hoc* comparisons, using Tukey Honest Significant Differences, were employed to test the effect of host species factorially. Using the vegan R package (Oksanen *et al.*, 2019), between species variation in beta diversity, at the individual ASV level, was investigated through ANOSIM-based community compositional dissimilarities and tests of multivariate homogeneity, employing Bray–Curtis dissimilarities to account for ASV relative abundances. Clustering was visualized through weighted PCoA (principal coordinates analysis), again using the vegan R package (Oksanen *et al.*, 2019). To investigate within species variation of beta diversity, the same methods were applied to a data subset including only Japanese macaques, employing host locality as the effector variable.

Results

Sequencing data consisted of 4 554 168 ITS-2 reads, with an average of 50 602 (S.D. 62 306) reads per sample. In total, 69 individual ASVs were identified, 65 of which were assigned to a single strongylid species: *Oesophagostomum aculeatum*. The remaining 4 ASVs were unidentified as strongylid taxa, assigned to superfamily trichostrongyloidea and could not be assigned at the genus level. The pairwise sequence distance (PSD) among all *Oesophagostomum* ASVs derived from the samples reached up to 10%, although such differences were restricted to 6 ASVs (8, 50, 51, 52, 57 and 67), with the PSD among all other ASVs not exceeding 5.4%. In the phylogenetic tree, all ASVs clustered within one strongly supported clade corresponding to *O. aculeatum*, with further internal structure (Fig. 1). One subclade comprised ASVs found in Bornean orangutans at both localities in Sabah, with 2 of these ASVs also detected in Japanese macaques in Jigokudani. Moreover, GenBank sequences originating from orangutans, in both Borneo (*P. pygmaeus*) and Sumatra (*P. abelii*), and Bornean white-bearded gibbons (*Hylobates albibarbis*) clustered within this subclade. The PSD within this subclade was below 2.5%.

Another small subclade comprised 5 ASVs (7, 9, 20, 23 and 30) detected in Japanese macaques across the 3 localities and one ASV (1) which occurred at all 3 Japanese localities as well as in crab-eating macaques at LKWS and orangutans at DVCA. This subclade also included 3 additional GenBank sequences from Japanese macaques. Other ASVs occurred across localities and host species, some occurred in multiple hosts at multiple localities, e.g. ASV 2 or ASV 22, some occurred in multiple hosts at a single locality, e.g. ASVs 25, 10 or 14, and some occurred in a single host at a single locality, e.g. ASVs 38, 27 or 69. Interestingly, these single-host-ASVs were always recorded in crab-eating macaques or Japanese macaques. It is worth mentioning that 2 sequences labelled as *O. bifurcum* in GenBank and originating from free-ranging bonobos (*Pan paniscus*) (Medkour *et al.*, 2021) clustered in the *O. aculeatum* clade and differed by only 1.2–6.6% (below 3.7% for most haplotypes) from any *O. aculeatum* sequence.

Of the 4 ASVs assigned only to trichostrongyloidea, all were restricted to a single host species at a single locality, occurring only in crab-eating macaques at LKWS, with none showing a close identity match with any GenBank sequences. All 4 ASVs clustered together and formed a small subclade (SUP FIG 1) closely related to a clade comprising *Hyostrongylus* spp. and to a clade including *Ostertagia nianqingtanggulansis* (AJ577459, from unspecified host from Tibet), *Ostertagia* sp. (AB367797, Japanese Serow, Japan), *Mazamastrongylus dagestanica* (JQ925868, OM445254; moose, Russia) and *Spiculopteragia* spp. (European cervids). Although *M. dagestanica* is currently assigned to the family Trichostrongylidae, all other taxa are from the family Haemonchidae, suggesting the nature of the trichostrongylids detected in macaques.

Upon visual inspection, alpha diversity (at the level of individual ASVs) of strongylid communities appeared to show minimal variation between host species (Fig. 2). GLMMs found statistically significant differences in alpha diversity with *post hoc* analysis revealing differences between only Japanese macaques and other host species, with no alpha diversity differences found among sympatric host species at LKWS ($P > 0.08$ for all pairwise comparisons). Japanese macaques showed lower alpha diversity of strongylid communities compared to crab-eating macaques, silvery lutungs and orangutans ($P < 0.001$ for all pairwise comparisons),

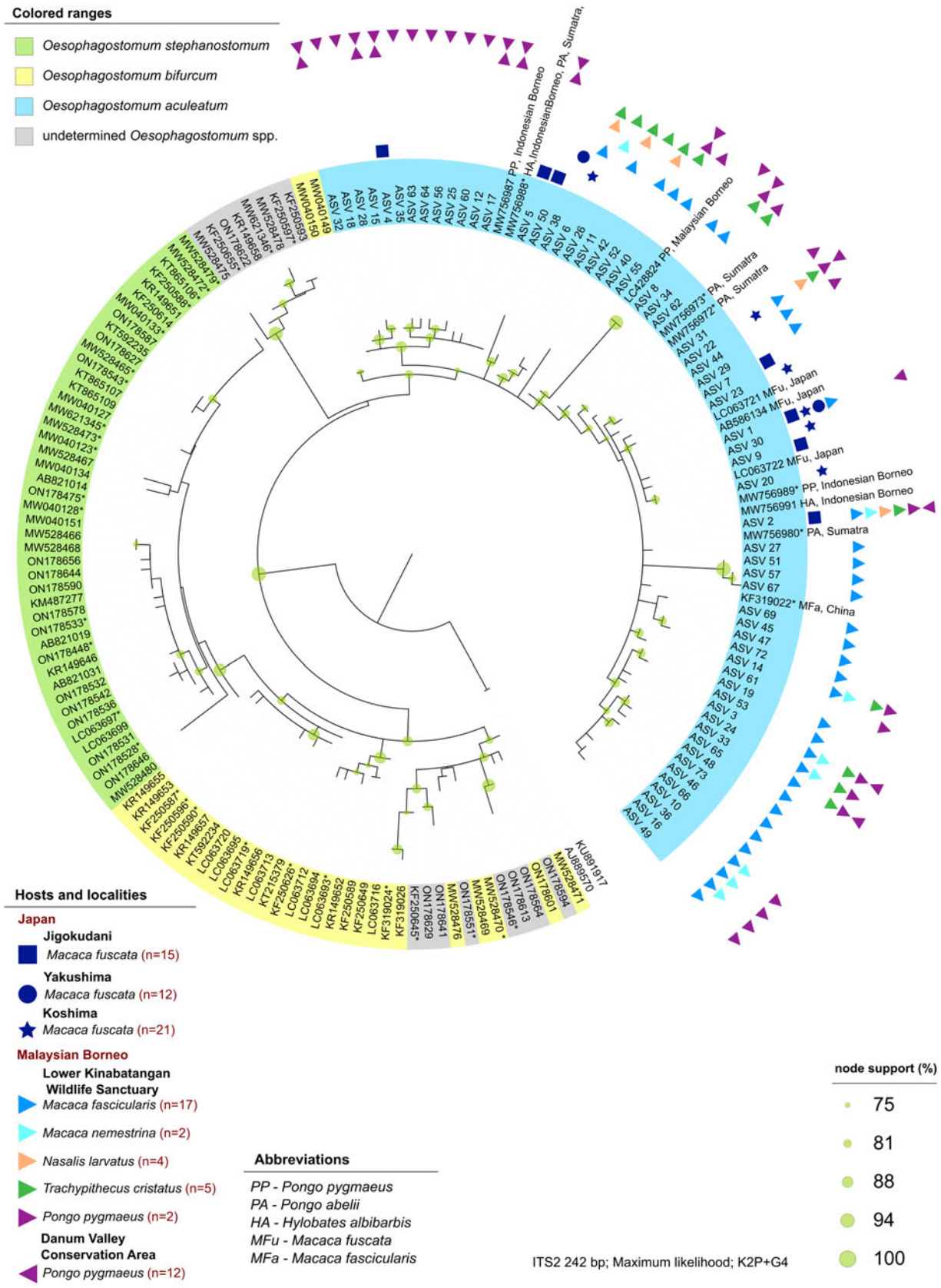


Figure 1. Maximum likelihood cladogram of *Oesophagostomum* ITS-2 region (245 bp), computed in IQ tree3 by model K2P + G4, using *O. dentatum* as an outgroup. The tree topology was tested by 1000 replicates of ultrafast bootstrap4 and SH-like aLRT5. Green circles mark nodes with support higher than 75%, with circle size depicting value. Sequences from GenBank are labelled by accession number.

yet no differences were found between the 3 localities in Japan. However, ASV composition of strongylid communities showed significant interspecific variation, both between host species (ANOSIM: $R = 0.597$, $P = 0.001$) and amongst Japanese macaques in different localities (ANOSIM: $R = 0.425$, $P = 0.001$), supported by visual inspection of ordination plots (Fig. 3).

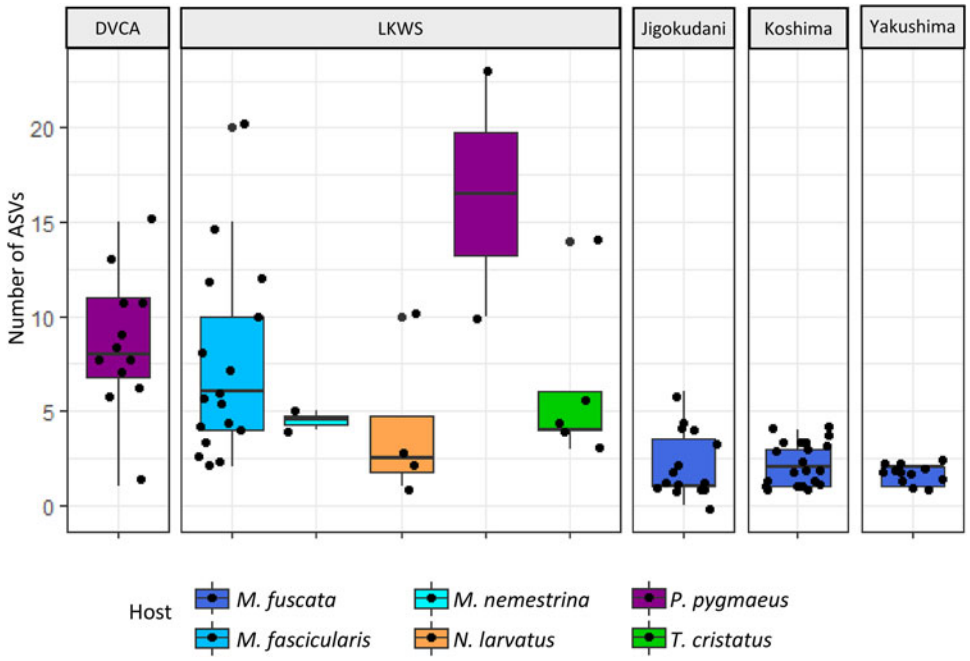


Figure 2. Amplicon sequence variant (ASV) diversity of strongylid nematodes detected in faecal samples of South-East and East Asian primates from 5 localities, indicated across the upper x-axis.

Discussion

Faecal samples from 6 NHP species inhabiting regions of South-East and East Asia were examined to assess the diversity of their strongylid nematode communities. A community profiling approach was applied, based on a previously optimized and tested HTS amplicon sequencing methodology. Focusing on strongylid communities from multiple NHP species allowed the diversity of these communities to be investigated with reference to sympatric species, different localities and host phylogenies. The reported diversity of strongylid nematodes highlights the potential consistency of these communities in NHP hosts within South-East and East Asia. The limitations of HTS methods should be noted, most poignantly the restriction in taxonomic resolution due to short sequence lengths and potential for sequencing errors from PCR biases (Kircher and Kelso, 2010; Ambardar *et al.*, 2016). However, previous work (Pafčo *et al.*, 2018) has shown successful approaches for diminishing the impact of these stochastic effects.

There was a clear dominance of *O. aculeatum,* with nearly all ASVs assigned to this taxon. Dominance of *O. aculeatum* was expected, mirroring previous reports of strongylid infections in Asian NHPs (Frias *et al.*, 2019). Still, the near complete absence of other strongylid taxa was surprising, as strongylid nematodes occur in complex communities comprised of multiple genera in other studied hosts. The majority of studied samples showed single species infections, contrasting with reports of strongylid infections in African NHPs (Pafčo *et al.*, 2019; Mason *et al.*, 2022; Ilík *et al.*, 2023). Previously implemented molecular methods, involving individual larval specimens, identified the presence of a single larva of *Ternidens* in one Bornean orangutan (Frias *et al.*, 2019), raising questions as to why this study did not identify the presence of *Ternidens*. The absence of *Necator americanus* among these samples should also be noted, as this strongylid has previously been reported in the local human population (Lim-Leroy and Chua, 2020) and is known to be frequently transmitted between humans and wild NHPs in Africa (Pafčo *et al.*, 2019). Potentially, this study is restricted by the limited sample size of some host species (especially orangutans at LKWS), with further sampling required for a more complete picture of strongylid diversity among Asian NHPs.

ASVs of *O. aculeatum* found in this study were further separated into 2 clusters. The first cluster consisted mainly of ASVs from orangutans, with 2 additional ASVs identified from Japanese macaques. This cluster also includes 4 sequences from GenBank, recorded as infecting *P. pygmaeus*, *P. abelii* and *H. albibarbis*, suggesting a lineage specialized to orangutans, with historical spill-over to other hosts. This cluster assembled closely with the 2 sequences from bonobos registered as *O. bifurcum*, though based on clustering these sequences may in fact represent *O. aculeatum* (Medkour *et al.*, 2021). The second cluster contained more ASVs and is identified in a larger diversity of host species, indicating it may be a more generalist lineage. Differentiation of these clusters as lineages or species would require morphological examination of adult worms and analysis of more markers. Cryptic species of *Oesophagostomum* have previously been proposed for nodular worm infections in humans and NHPs in Uganda (Ghai *et al.*, 2014), with high genetic variability of *Oesophagostomum* spp. observed in African primates (Sirima *et al.*, 2021; Mason *et al.*, 2022). However, although the ITS-2 marker, also used in this study, is ideal for distinguishing between known species for which genetic data exists in repositories, it should be noted that this marker shows high levels of intraspecific variability, which may limit its value in identifying potential cryptic species (Conole *et al.*, 1999; Poissant *et al.*, 2021; Halvarsson and Tydén, 2023). Several host-specific lineages were previously found in *Oesophagostomum* following *cox1* analyses with 2 clear lineages showed within *O. aculeatum*, the first being found in a number of hosts, while the second being more host-specific (Frias *et al.*, 2019). This confirms a certain degree of host preference within the *O. aculeatum* lineages detected and shows that involving genetic markers of divergence at the mitochondrial DNA loci would be an important aid in deciphering potentially cryptic species (Blouin, 2002).

The 4 trichostrongyloidea ASVs identified in crab-eating macaques highlight the current convolution in genetic distinction of strongylids in wildlife hosts. There are records of unspecified

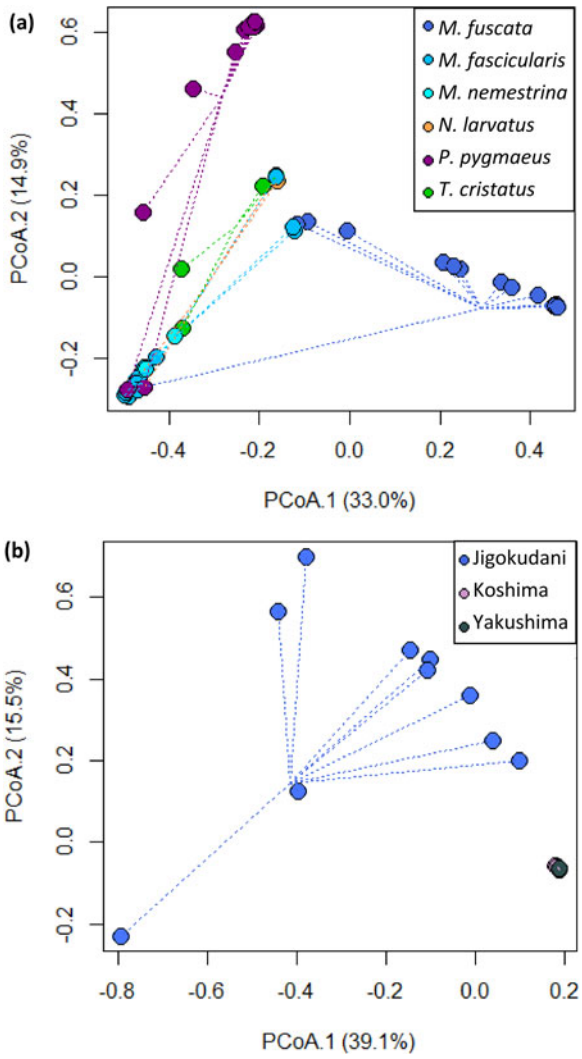


Figure 3. Principal coordinate analysis (PCoA) plots of the beta diversity (assessed through Bray–Curtis dissimilarities) among strongylid nematode communities of (a) 6 South-East and East Asian Primate species from 5 localities and (b) *M. fuscata* from 3 localities in Japan. Convergent dots indicate similarities in community composition. While Koshima and Yakushima are represented by multiple samples, close convergence of points shows no separation within ordination space at this scale.

Trichostrongylus sp. based on the presence of eggs in various Asian macaque species (Kumar *et al.*, 2018; Kurniawati *et al.*, 2020; Fernando *et al.*, 2022). Although humans and some African cercopithecoid primates are occasionally reported with *Trichostrongylus colubriformis* infection (Phosuk *et al.*, 2013; Sharifdini *et al.*, 2017; Obanda *et al.*, 2019), and apparently there are *Trichostrongylus* spp. infecting African great apes (Mason *et al.*, 2022), these sequences did not cluster with any *Trichostrongylus* spp. provided in the GenBank. Thus, as a more probable candidate *Nochtia nochti* is proposed, a trichostrongyloid nematode parasitizing *Macaca* spp. across Asia which unfortunately is not represented in available sequence databases. However, pathogenesis described in *N. nochti* (Yalcindag *et al.*, 2021; Fonti *et al.*, 2023) holds similarities to other trichostrongyloids from the family Haemonchidae (Deplazes *et al.*, 2016), within which these sequences clustered.

The number of strongylid ASVs per sample, a quasi-measure of alpha diversity, was generally consistent across host species. Of all host species, only Japanese macaques showed noticeable differences (significantly lower number of ASVs) from other species. This finding may be as expected, with Japanese macaques being the unique sampled species occurring in East Asia, compared to the other species in South-East Asia. Of particular note is the variation identified between the 2 macaque species, Japanese macaques and crab-eating macaques, highlighting how geographical factors and sympatric species can influence strongylid communities of closely related host taxa. Japanese macaques are the world's most northern-living primate (Cozzolino *et al.*, 1992; Tsuji *et al.*, 2008), meaning it is one of the few NHP species that exists solely in single-primate communities (Ito *et al.*, 2021). As such, the ability for parasites to host switch, a method whereby a parasite establishes within a novel host species (De Vienne *et al.*, 2007; Cooper *et al.*, 2012), is limited due to the absence of closely related species already hosting the parasite. Fossil evidence suggests that Japanese macaques may have long existed within a single-primate community (Marmi *et al.*, 2004; Kawamoto *et al.*, 2007), which may also be reflected among their intestinal parasite fauna (Gotoh, 2000).

Additionally, this northern distribution means Japanese macaques inhabit Japanese islands where environmental conditions differ quite dramatically from those in Borneo. Japanese macaques living at more northern latitudes, where the climate is characterized by colder winters, were previously shown to carry fewer gastrointestinal parasites at lower prevalence (Gotoh, 2000). The harsher seasonal environment in Japan may reduce the longevity of strongylids during developmental stages in the external environment (Knapp-Lawitzke *et al.*, 2014). As such, ASV diversity, in Japan compared to Borneo, is limited by the robustness of strongylid taxa and their ability to withstand harsher environmental conditions.

Great variation between the samples according to the community composition of strongylids was observed both among species as well as within species (when concerning Japanese macaques). Some of this among-species variation is explained by the 2 identified lineages of *O. aculeatum*, with the divergent ordination of orangutans coinciding with the '*orangutan*' lineage, almost exclusively identified within this host. Likewise, the divergent ordination of crab-eating and pig-tailed macaques from Japanese macaques attests to the importance of sharing a habitat with other sympatric primates, supported by the widespread second lineage of ASVs identified within these hosts. Japanese macaques occupying a separate ordination space are additionally at least in part due to the reduced diversity of strongylid communities in this host species. This divergent ordination of macaque species, suggesting divergent composition of strongylid communities, contradicts the host–parasite co-speciation hypothesis (Brooks, 1979). Interestingly, only a single ASV was commonly identified among all 3 macaque species, with Japanese macaques sharing just 3 additional ASVs with either crab-eating or pig-tailed macaques, highlighting the role of environmental factors influencing parasite communities. Divergent ordination among Japanese macaques from different localities further emphasizes this, with hosts of the same species showing variation within strongylid community composition and only a single shared ASV identified across all 3 localities. Geographical isolation within Japanese macaques has led to the evolution of a subspecies in one locality: *M. f. yakui* in Yakushima (Hayaishi and Kawamoto, 2006). Host evolution is not mirrored by their strongylid communities in this case, however, with Yakushima strongylid communities showing convergence, in terms of composition, with Koshima, while Jigokudani strongylid communities show far greater divergence, again opposing the host–parasite co-speciation hypothesis. Further exploration of this hypothesis among NHPs, and environmentally driven deviation, could benefit from using the *Macaca* genus as a model, with 22 currently recognized species distributed into several lineages (Thierry, 2007) and occupying the widest geographical range of all NHP, spanning both tropical and temperate regions (Fooden, 1982).

Concluding, the strongylid nematode communities of South-East and East Asian NHP were dominated by *O. aculeatum*, with single-species occurrence identified in most samples, which is in sharp contrast to the rich strongylid communities harboured by African great apes and other African NHPs. The genetic distances between sequences suggests the potential for 2 cryptic lineages within *O. aculeatum*. One lineage appears more specialized in terms of host diversity, with the other being more generalist and diverse. However, ITS-2 does not provide a suitable marker for evaluation of intraspecific diversity, therefore further sequencing with the inclusion of more genetic markers is required to better decipher the genetic make-up of the *O. aculeatum* complex. These findings highlight how geographic variables may influence parasite communities within NHPs, disuniting host–parasite co-speciation. While the reported strongylid communities seem low in species richness, this report is only an initial insight and additional sampling is required to truly capture the genetic diversity of strongylid communities in Asian NHPs. Establishing baseline data on NHP strongylid infections can assist with ongoing monitoring of health threats to wild NHPs with HTS providing complex insights into strongylid community composition and ability to detect even less prevalent, but more pathogenic taxa.

Supplementary material. The supplementary material for this article can be found at https://doi.org/10.1017/S0031182024000386.

Data availability statement. Strongylid ITS-2 sequencing data is archived in the European Nucleotide Archive, under the accession number of the whole project PRJEB73875 (available at: https://www.ebi.ac.uk/ena/browser/view/PRJEB73875). Accession numbers for each sample are available alongside related metadata in Supplementary Table 1, which includes geographical location and sampling period.

Acknowledgements. The authors extend gratitude to the Sabah Wildlife Department and Sabah Biodiversity Centre (SaBC) for allowing us to conduct this research through multiple access licences (JKM/MBS.1000-2/3 (101) to RM; JKM/MBS.1000-2/2 (280), JKM/MBS.1000-2/2 JLD.3 (106), JKM/MBS.1000-2/2 JLD.5 (82), JKM/MBS.1000-2/2 JLD.6 (121) to LF; JKM/MBS.1000-2/2 JLD.3 (35), JKM/MBS.1000-2/2 JLD.5 (68), JKM/MBS.1000-2/2 JLD.6 (108) to AJJM) and sample export licences (JKM/MBS.1000-2/3 JLD.2 (62) to AJJM; JKM/MBS.1000-2/3 JLD.3 (108) to SP), as well as to Yayasan Sabah for permission to work at DVCA. We also thank Noko Kuze, Tomoko Kanamori, Henry Bernard and Peter Malim for supporting research at DVCA. We thank the Cooperative Research Programme of the Wildlife Research Center and Kushima City for permissions to conduct research at Koshima, while also thanking the Cooperative Research Programme of the Wildlife Research Center, Yakushima Forest Office and Kagoshima Prefecture for permission to conduct research on Yakushima. Additionally, we thank Jigokudani Monkey Park for permission to conduct research there.

Author contributions. AJJM, BP and KJP conceived and designed the study while LF, BG, KK, AL, RM, SP, SS, ZX and AJJM collected and/or facilitated sample collection. BM, KK, AL, KMaj and ZX undertook initial laboratory work, under supervision of MO, BP and AJJM. HH, KK, AL, TM, KMaj, KMat, ZX and BP were responsible for sequencing work, with the resulting data then analysed by BM, BC and BP. BM wrote the article initially, with supervision from BP, AJJM and KJP, before all co-authors reviewed and improved the article.

Financial support. We are extremely grateful to the Operational Programme Research, Development and Education – Project Internal Grant Agency of Masaryk University (No. CZ.02.2.69 / 0.0 / 0.0/ 19_073/ 0016943) for supporting this work and in particular funding BM's involvement. We are also grateful for the support from Japan Society for the Promotion of Science (JSPS)-Czech Academy of Science (JSPS-CAS) bilateral cooperative programme, Japan/Czech Republic (project Nos. JPJSBP120192506 and JSPS-19-17) and from the Czech Science Foundation (22-16475S). We extend further gratitude to JSPS for supporting our research in Borneo, through grants awarded to AJJM (#24770232, #20H03333, #16H06181), MO (#15H04283) and LF (#446). The study was further supported through Kyoto University's Step-Up Program awarded to AJJM. Finally, we thank the Cooperative Research Programme of the Kyoto University Primate Research Institute, for supporting SP's involvement.

Competing interests. The authors declare there are no conflicts of interest.

Ethical standards. Authorization to conduct research and collect biological samples in Sabah was granted by the Sabah Biodiversity Centre (SaBC) and Yayasan Sabah, with the support of the Sabah Wildlife Department. Our field protocols strictly followed the guidelines established by these agencies, as well as the regulations set by the Field Research Committee at the former Kyoto University Primate Research Institute, Japan.

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Pafčo, B., Nosková, E., Ilík, V., Anettová, L., Červená, B., Kreisinger, J., Pšenková, I., Václavek, P., Vyhlídalová, T., Ježková, J., Malát, K., Mihalca, A.D., Modrý, D.

2024

First insight into strongylid nematode diversity and anthelmintic treatment effectiveness in beef cattle in the Czech Republic explored by HTS metagenomics.

Vet. Parasitol. Reg. Stud. Rep. 47, 100961. https://doi.org/10.1016/j.vprsr.2023.100961.

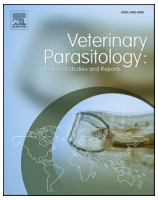
Veterinary Parasitology: Regional Studies and Reports 47 (2024) 100961



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Regional report



First insight into strongylid nematode diversity and anthelmintic treatment effectiveness in beef cattle in the Czech Republic explored by HTS metagenomics

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ARTICLE INFO

Keywords:
Cattle
Strongylid nematodes
Resistance
Anthelmintic treatment

ABSTRACT

Parasitic diseases and mitigation of their effects play an important role in the health management of grazing livestock worldwide, with gastrointestinal strongylid nematodes being of prominent importance. These helminths typically occur in complex communities, often composed of species from numerous strongylid genera. Detecting the full diversity of strongylid species in non-invasively collected faecal samples is nearly impossible using conventional methods. In contrast, high-throughput amplicon sequencing (HTS) can effectively identify co-occurring species. During the four-year project, we collected and analysed faecal samples from beef cattle on >120 farms throughout the Czech Republic. Strongylids were the predominant nematodes, detected in 56% of the samples, but at a low level of infection. The apparent limitations in identifying strongylid taxa prompted this pilot study on a representative group of samples testing positive for strongylids using ITS-2 metabarcoding. The most widespread genera parasitizing Czech cattle were *Ostertagia* (*O. ostertagi*) and *Oesophagostomum* spp., followed by *Trichostrongylus* and *Cooperia*, while *Bunostomum*, *Nematodirus* and *Chabertia* were present only in a minority. As comparative material, 21 samples of cattle from the Danube Delta in Romania were used, which, in contrast, were dominated by *Haemonchus placei*. Finally, the effect of ivermectin treatment was tested at two Czech farms. After treatment with the anthelmintic, there was a shift in the strongylid communities, with a dominance of *Cooperia* and *Ostertagia*.

1. Introduction

Helminth infections are considered a major cause of production and related financial losses in the livestock industry (Ploeger et al., 1990; Canton et al., 2020) with strongylid nematodes forming an important part. In Australia, for example, economic losses caused by these nematodes are estimated at one billion dollars, with additional tens of billions of dollars spent on their treatment (McLeod, 1995; Rashid et al., 2019). Long-term use of anthelmintics leads to an increase in anthelmintic resistance, making control more difficult and further increasing economic losses (Canton et al., 2020). The diversity and importance of strongylid nematodes and, in particular, the emergence of resistance to

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https://doi.org/10.1016/j.vprsr.2023.100961
Received 4 May 2023; Received in revised form 25 October 2023; Accepted 20 November 2023
Available online 8 December 2023
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anthelmintics have received considerable attention in the European Union, e.g., in Belgium, Germany, Sweden, and the United Kingdom, where parasite infections in cattle are a growing problem as they result in lower milk yield and weight gain (Charlier et al., 2010; McArthur et al., 2011). Neither helminthiases nor anthelmintic resistance in cattle are centrally monitored in the EU (Charlier et al., 2022). More detailed data are more than desirable, especially when addressing the issue of the emergence of resistance to anthelmintics and the assessment of the current infection situation.

Analyses of strongylid nematode diversity are complicated by the coexistence of multiple species in complex communities (Van Wyk et al., 2004; Lichtenfels et al., 2008). Even in cattle, a single host (or herd) is parasitized by a number of genera/species of strongylids (Avramenko et al., 2015; Francis and Šlapeta, 2022). Individual species differ in their location in the host, the nature of their pathogenicity, and their ability to be resistant to antiparasitic agents. The classical methods for detecting nematodes employed in cattle health management do not allow accurate identification of the nematode taxa involved, especially in coinfections with a larger number of species. Research on parasitic nematode diversity is shifting to DNA-based methodologies (Chilton, 2004), but the limited number of species that can be detected in mixed infections is a major weakness of Sanger sequencing-based methods. High-throughput sequencing methods (HTS) represent a real game changer as they allow the spectrum of strongylid nematodes to be sequenced and effectively described, including the relative abundance of helminth taxa involved (Avramenko et al., 2015, 2017; Pafčo et al., 2018).

To fill a gap in knowledge on diversity of strongylid nematodes parasitizing cattle in central Europe, we designed a pilot study and applied Illumina ITS-2 metabarcoding applied on a set of samples collected across the Czech Republic. To illustrate the usefulness of the approach for investigation of the geographic component of strongylid distribution across Europe, we compared strongylid nematode community in Czech cattle with a sample set from rural area of the Danube Delta in Romania. Last but not least, the diversity of nematodes before and after treatment with ivermectin was investigated in two farms. Considering the cross-border movement of cattle, this novel data on species composition of strongylid communities in beef cattle in the Czech Republic likely also reflect the situation in all of Central Europe.

2. Materials and methods

2.1. Sample collection

A baseline set of >8000 faecal samples from beef cattle from over 120 farms was collected within the project "Monitoring of strongylids involved in helminthiases of beef cattle in the Czech Republic and analysis of anthelminthic resistance and diversity". Most samples were collected by the farmers that received a packet of sampling equipment and methodology, including a questionnaire on the antiparasitic treatment used in each herd. Samples were collected as fresh as possible from pastures, stalls, or directly from the rectum during regular veterinary examinations and transported immediately to the project laboratories. Part of the sample was frozen immediately after receiving the shipment and stored at −20 °C for subsequent molecular analyses. All fresh samples were examined microscopically using standard sedimentation and flotation methods no later than 48 h after collection (Modrý et al., 2022). Although almost 60% of samples were positive for strongylid nematodes (Modrý et al., 2022), majority showed low infection intensities (~95% of samples 1–100 EPG).

For HTS pilot study we selected samples positive for strongylid eggs in microscopy ($n = 95$) from 18 farms, trying to cover all regions of the Czech Republic (Fig. 1). Additional information, including location, breed, sex/age category, and an estimate of infection intensity, is provided in the Appendix (Table S1). Using a semiquantitative method, samples from Czech cattle were divided into three categories: 66% of samples contained tens of EPG (1−100), 15% of samples contained

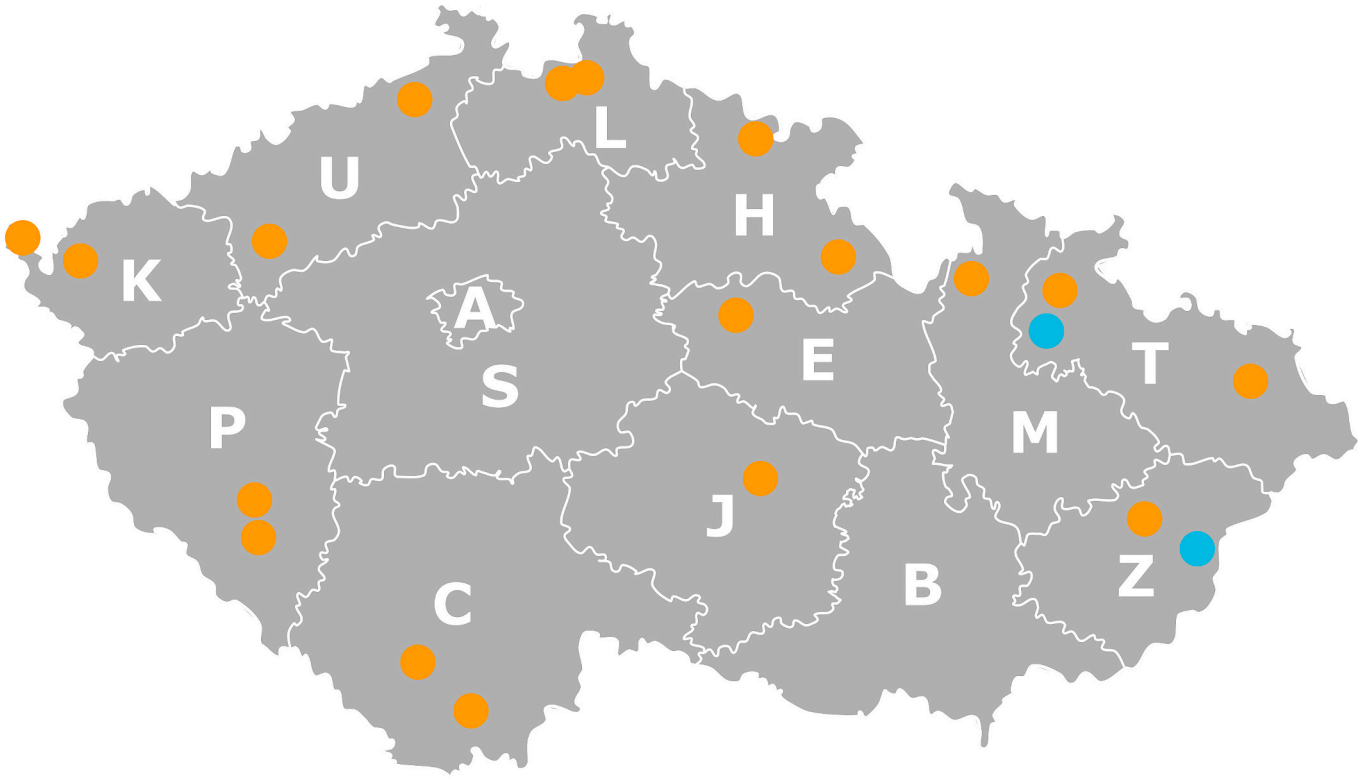


Fig. 1. Map showing locations of beef cattle farms included in the study. Farms included in the evaluation of the overall diversity of strongylid nematodes in the Czech Republic are shown in orange, while farms where the effect of treatment on strongylid nematodes was tested are shown in blue. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

lower hundreds (101–500), while only one sample contained over 500 EPG. A set of samples ($n = 21$) from geographically distant Romanian Dobrodgea (Tataru Island in the Danube Delta Biosphere Reserve) was included. In case of two Czech farms, we managed to sample beef cattle before and 14 days after anthelmintic treatment ($n = 46$ for Farm 1 and $n = 48$ for Farm 2) with ivermectin (applied s.c). Specifically, Noromectin (Norbrook, 1% solution) and Virbamec (Virbac, 1% solution) were used at Farm 1 and 2, respectively. Ivermectin is the most common treatment in the Czech beef cattle (data not shown), and therefore we were able to test the impact of the anthelmintic treatment on the nematode diversity and relative abundance. We used the Mini-FLOTAC© (Cringoli et al., 2017) to calculate the number of eggs per gram of faeces (EPG) before and after treatment for both farms. The Mini-FLOTAC© EPG sensitivity was set to five. Faecal egg count reduction (FECRT) was calculated to evaluate possible resistance to ivermectin treatment, where each animal served as its own control (Cabaret and Berrag, 2004).

2.2. DNA isolation and ITS-2 nemabiome metabarcoding

Total genomic DNA was extracted from the individual frozen faecal samples (95 samples of Czech cattle across the country, 21 samples of Romanian cattle and 90 samples from two Czech farms collected before and after anthelmintic treatment) using PowerSoil DNA isolation kit (Qiagen Company, Germany). PCR amplification was performed on the second internal transcribed spacer (ITS-2) of nuclear ribosomal DNA with forward primer Strongyl_ITS-2_F (acg tct ggt tca ggg ttg) and reverse Strongyl_ITS-2_R (atg ctt aag ttc agc ggg ta). We have already showed that these primers exhibit a low level of PCR bias and provide consistent community profiles (Pafčo et al., 2018), suggesting that they can be used for semiquantitative analyses of complex strongylid communities. HTS sequencing libraries were generated using a two-step-PCR approach following Nextera primer design (Pafčo et al., 2018). Analyses were carried out in two technical replicates with different tag primer barcodes. DNA extraction from strongylid negative faeces and water were used as negative controls and synthetic DNA templates as positive controls (Pafčo et al., 2018). The final library was sequenced using MiSeq Reagent Kit v3 (2 × 300 bp pair end reads 600 cycles) by Illumina MiSeq platform.

2.3. Bioinformatic data processing and statistics

We trimmed gene-specific primers using skewer (Jiang et al., 2014). Low quality (expected error rate > 1%) reads were eliminated, and the filtered data set was denoised using dada2 (Callahan et al., 2016). To avoid inflation of strongylid diversity by PCR/sequencing artefacts (that were not corrected by dada2) in downstream analyses, we kept only those sequences / ASVs (amplicon sequencing variants) that were consistently present in both technical duplicates for a given sample (Pafčo et al., 2018). Next, AssignTaxonomy method implemented in dada2 was used for taxonomic assignation of ITS-2 ASVs using Nematode ITS-2 reference database (Workentine et al., 2020). Minimum number of the reads for the sample was set to 200 (Halvarsson and Höglund, 2021), however all samples passed the filtering.

The bar charts, dot plots and boxplots displayed in figures were created in R Studio version 0.99.489. Statistical analyses focused on variation in strongylid community composition before and after the anthelmintic treatment application. We first investigated variation of alpha diversity, through the number of different ASVs per sample (number of ASVs per sample = species richness). We then tested the effect of treatment on ASVs diversity using Wilcoxon test (stats R package; R Core Team, 2013). We investigated differences in beta diversity before and after the treatment application through ANOSIM-based community compositional dissimilarities (vegan R package; Oksanen et al., 2019), defined as prevalence and relative representation of ASVs. We visualised clustering with Principal Coordinate Analysis (PCoA) for Bray-Curtis Dissimilarities (McMurdie and Holmes, 2014), accounting for ASV relative abundances, as well as a binary version of Jaccard Index, considering only ASV presence/absence (phyloseq R package; McMurdie and Holmes, 2013).

3. Results

After quality filtering and elimination of samples where the sequencing depth was low, we gathered ITS-2 profiles for all 116 samples (100%) for evaluating the strongylid diversity (samples from Czech and Romanian cattle) and 70 samples (~ 78%) for the treatment effectiveness evaluation. Sequencing data for strongylids ITS-2 are archived in the European Nucleotide Archive under the accession project number (PRJEB67745), available at https://www.ebi.ac.uk/ena/browser/view/PRJEB67745, accession numbers for each sample are available in supplement (Table S1, S2). Our sequencing data comprised 7,221,006 (averaging 38,822 per sample) ITS-2 high quality reads.

3.1. Strongylid nematode in beef identified at the species level

In total, 144 strongylid nematode ASVs from eight genera and 12 species were identified in the strongylid nemabiome analyses (Fig. 2). All but six strongylid ASVs were identified to species level. In individual hosts, strongylid nematodes were found in complex communities composed of multiple species - based on ASVs in both localities, the majority of samples contained six ASVs, but after clustering ASVs at the species level, most samples in the Czech Republic and Romania contained two species (33% and 57%, respectively), followed by three species (24% and 30%, respectively), one species (25% and 5%, respectively), or four species (9% and 14%, respectively), while the maximum was six species of strongylid nematodes, found in only 1.2% of samples in cattle in the Czech Republic. At the genus level, the data set from Czech cattle was dominated by *Ostertagia*, followed by *Oesophagostomum*, *Trichostrongylus* and *Cooperia*, while the genera *Bunostomum*, *Nematodirus*, and *Chabertia* were represented to a lesser extent (Fig. 2). In comparison, *Haemonchus* (*Haemonchus placei*) dominated the samples from Romania, followed by *Trichostrongylus* and *Ostertagia* while *Oesophagostomum* and *Cooperia* were in the minority. In the Czech samples, *Ostertagia ostertagi*, *Cooperia oncophora*, *Oesophagostomum radiatum* and *Trichostrongylus axei* were the most prevalent species found (Fig. 2).

3.2. Ivermectin administration impacts the strongylid community

In two farms, sample sets collected before and after ivermectin treatment were evaluated separately. Firstly, the number of eggs per gramme of faeces (EPG) was calculated for all animals at both farms before and after treatment with anthelmintics. The mean value before treatment was 21 (standard errors ±19) EPG and 2.2 (±2.9) EPG after treatment at Farm 1, while 113 (±101) EPG before and 4.6 (±19) EPG after treatment at Farm 2 (Fig. 3A, Table S2). Although the EPG was already low before the treatment, we calculated the egg count reduction (FECRT). The FECRT after treatment with ivermectin was 83.3% at Farm 1 indicating possible resistance, whereas 98.6% recorded at Farm 2 shows susceptibility of strongylids to ivermectin. However, none of the pre-treatment mean values exceeded EPG ≥ 150.

Second, using ITS-2 metabarcoding, we found 119 ASVs belonging to eight genera and 10 species. All but 14 ASVs were assigned to the species level. The number of positive samples decreased on both farms (Farm 1: 22 sample before to 14 samples after and Farm 2: 23 samples before to 11 samples after treatment; Fig. 3B). Although the strongylid nematodes did not completely disappear in tested animals from these two herds, there was a clear shift in their spectrum (Fig. 3B). In the Farm 1, *Haemonchus contortus* and *Nematodirus helvetianus* completely disappeared, *Ostertagia ostertagi* declined significantly, while *Cooperia oncophora* dominated the samples after the treatment. In the Farm 2, there was a significant reduction in *Oesophagostomum radiatum*, *Bunostomum phlebotomum* and *Trichostrongylus axei* in favour of *Ostertagia ostertagi* and

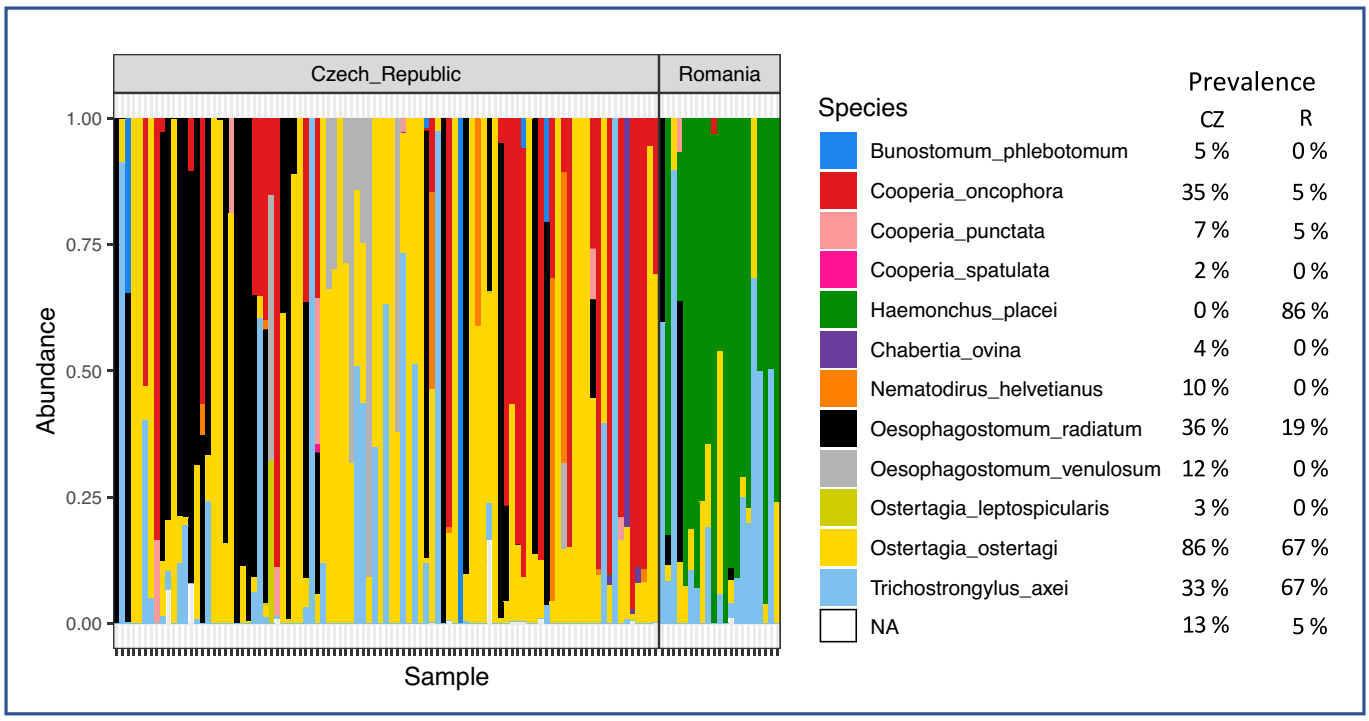


Fig. 2. Bar-plot shows community composition of strongylid nematodes at species level together with percentage of positive sample (prevalence) from all samples analysed in representative set of faecal samples from beef cattle in the Czech Republic and Romania.

two *Cooperia* species. The number of strongylid ASVs per sample (alpha diversity) significantly differed before and after the treatment application ($p > 0.001$; Fig. 3C). Visual inspection of PCoA ordination plots show only mild clustering of samples before and after the treatment application with Bray-Curtis dissimilarities (Fig. 3C), however, strongylid communities composition showed significant interspecific variation before and after the treatment application (ANOSIM - Jaccard Index: $R = 0.265$, $p = 0.001$; Bray-Curtis Dissimilarities: $R = 0.248$, p = 0.001).

4. Discussion

High-throughput sequencing has proven to be an effective tool for assessing complex communities, including strongylid nematodes ITS-2 metabarcoding in ruminants and other herbivores (Avramenko et al., 2015; Pafčo et al., 2018; Halvarsson and Höglund, 2021; Francis and Šlapeta, 2022; Halvarsson et al., 2022). However, HTS data describing the diversity of strongylid nematodes in cattle are only available from America (Avramenko et al., 2015, 2017) and Australia (Francis and Šlapeta, 2022). Parasite surveillance, including correct and timely application of diagnostic methods, is crucial for effective parasite control and limiting the spread of anthelmintic resistance, however, comprehensive data on strongylid diversity at the species level in cattle have been lacking in the Czech Republic. Our study provides the first DNA-based HTS insight into the diversity of strongylid gastrointestinal nematodes in beef cattle not only in the Czech Republic, but throughout Europe.

This study is part of a four-year surveillance of helminths in beef cattle in the Czech Republic, that aimed at comprehensive analysis of bovine parasites. Briefly, strongylid gastrointestinal nematodes were the most common parasites found during surveillance in all but one farm (Modrý et al., 2022), which is consistent with previous studies from the Czech Republic that identified strongylid eggs using microscopy and the major strongylid genera based on L3 larval morphology (Kváč, 2003; Chroust, 2006; Hromadová, 2012; Kubelka, 2016). Given the massive cross-border movement of cattle in recent decades, it is not surprising that the situation is similar to other European countries (Stancampiano et al., 2007; Taylor, 2010; Theodoropoulos et al., 2010).

Our ITS-2 metabarcoding data show a dominance of *Ostertagia ostertagi*, followed by other trichostrongylids, but also genera with a lower proportion such as *Bunostomum*, *Nematodirus*, and *Chabertia*. At the genus level, our results agree well with historical data from studies based on L3 morphology, when strongylid nematodes in cattle were dominated by the genus *Ostertagia*, followed by *Cooperia*, *Trichostrongylus*, *Oesophagostomum*, and *Nematodirus* (Chroust, 1982, 2006). *Ostertagia*, along with *Cooperia*, are the most abundant strongylids in cattle worldwide, and *O. ostertagi* is also the most economically important strongylid pathogen in cattle (Charlier et al., 2012). The ubiquitous occurrence of the latter species in cattle herds in the Czech Republic indicates the need for continuous surveillance, notwithstanding the very low EPGs detected in our sample (mostly tens of eggs per gramme). This is also consistent with previous results that reported the highest egg numbers in summer but never exceeded 150 EPG (Chroust, 2006), as seems to be the case elsewhere (Geurden et al., 2015; Avramenko et al., 2017).

Several million cattle are moved within the EU annually (Dahl-Pedersen and Herskin, 2023), making the introduction of new parasite species possible (Short et al., 2017). Knowledge of strongylid diversity, i.e., the application of metabarcoding, is a useful tool for monitoring and explaining regional variation at different scales, including transboundary animal movements (Avramenko et al., 2017). Our results demonstrate a significant difference in strongylid community composition between the Czech cattle herds and a comparative set of samples from Dobrodgea, Romania. In the latter area, the strongylid community was clearly dominated by *Heamonchus placei*, a pathogenic and economically important bovine nematode (Fávero et al., 2016). Although *H. placei* is a parasite of cattle primarily in tropical and subtropical regions, it has also been found in temperate climates with cold winters (Jabbar et al., 2014). In contrast, in the Czech sample set, we found only *H. contortus* in a single farm before treatment. This strongylid is usually found in small ruminants but has also been detected in cattle (Achi et al., 2003; Amarante et al., 1997).

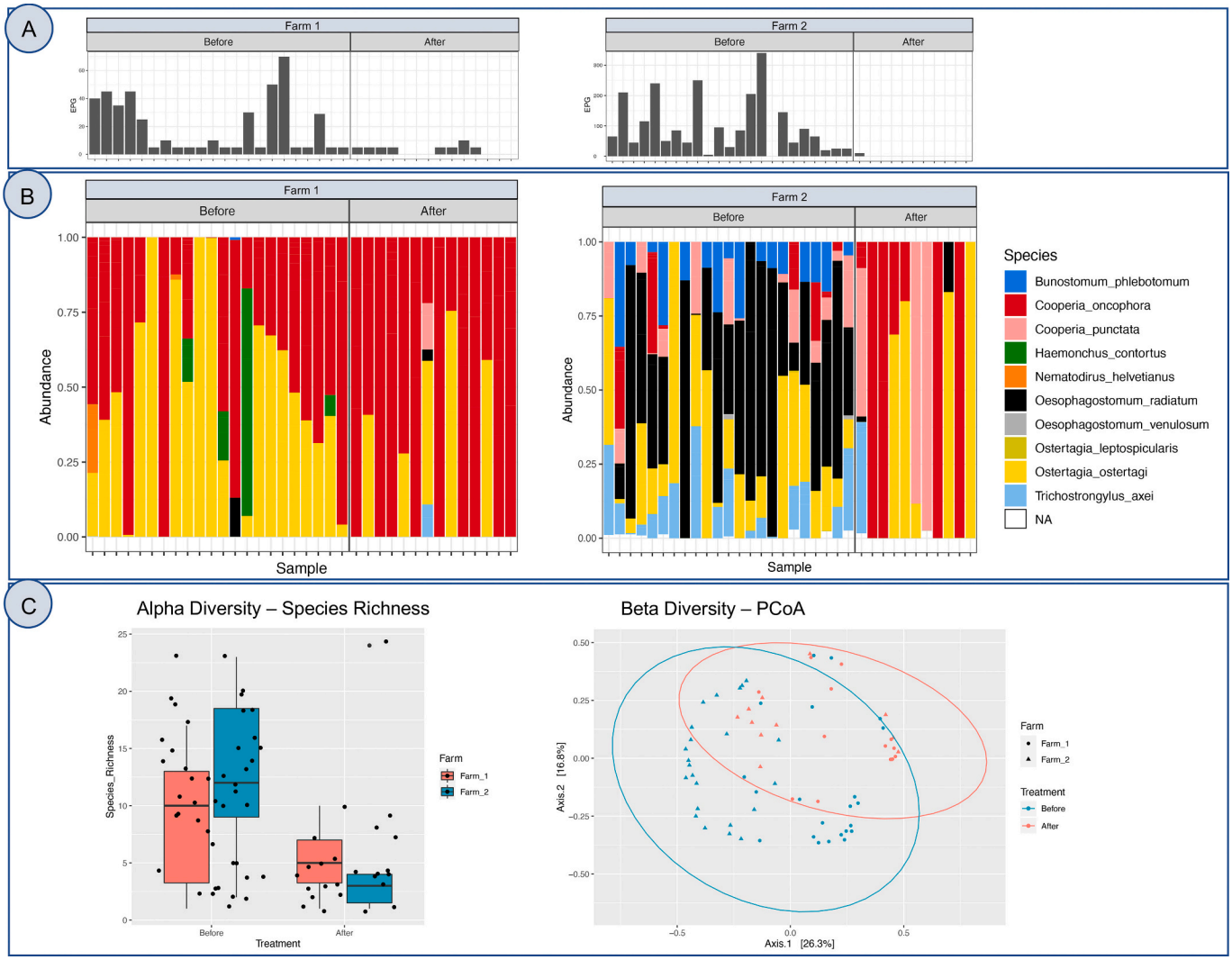


Fig. 3. Bar-plot shows A) faecal egg counts and B) community composition of strongylid nematodes at species level (each column represents the relative abundance of ITS-2 amplicon sequence variants per sample) and C) amplicon sequence diversity (Species Richness) and Principal coordinate Analyses (PCoA) plots of beta diversity based on Bray-Curtis dissimilarities of strongylid nematodes in beef cattle in the Czech Republic before and after anthelmintic treatment.

Resistance of strongylid nematodes to macrocyclic lactones and benzimidazoles is a growing problem worldwide (Sutherland and Leathwick, 2011); macrocyclic lactones are the most commonly used anthelmintics in cattle in the Czech Republic (Modrý et al., 2019). On a limited scale, we had the opportunity to study the effect of macrocyclic lactones on strongylid nematodes in two farms and to show changes in strongylid diversity after treatment. In both farms, the number of positive samples decreased and some of the strongylid species disappeared or were significantly reduced after ivermectin treatment. When evaluating alpha and beta diversity, we noted a decrease in ASVs after treatment, indicating a significant reduction of the strongylid nematode population and thus at least partial efficacy of ivermectin at the farms studied. We also observed differences in beta diversity before and after treatment, caused by an overall reduction in diversity in favour of a few dominant species persisting after anthelmintic application; more specifically, *Ostertagia* (Farm 1) and *Cooperia* (both farms) dominated after the treatment, which may indicate the onset of resistance to anthelmintics. Similar changes in the species composition of nematode communities were described in Canadian beef farms (Avramenko et al., 2017). *Cooperia* spp. are strongylids most commonly reported to be resistant to anthelmintics (Gasbarre et al., 2009; El-Abdellati et al., 2010; Bartley et al., 2012) and resistance has also been demonstrated in *Ostertagia ostertagi* (Edmonds et al., 2010; Avramenko et al., 2017). Considering the pathogenicity of *O. ostertagi*, the potential increase in anthelmintic resistance could have significant negative consequences for cattle management. On the other hand, the FECRT results did not confirm resistance on Farm 2, where the EPG was significantly reduced after the ivermectin treatment. Nevertheless, it is important to note that the EPG was already low (mean 21 and 113 EPG in Farm 1 and 2, respectively) and therefore the results should be regarded with caution.

5. Conclusion

Metabarcoding of nemabiome using high-throughput sequencing has the potential to make a fundamental contribution to our understanding of the species composition of strongylid nematodes, the efficacy of targeted nematode control, and, in particular, the prevention of anthelmintic resistance. We provide the first data on species composition of strongylid diversity in cattle in the Czech Republic, but also in Europe as a whole. Although species richness in individual samples was not high, we identified 12 nematode taxa and demonstrated that multiple species can be present in a single sample, with *Ostertagia ostertagi* clearly dominating. In two farms sampled before and after anthelmintic treatment, we noted a decrease in strongylid nematodes and a shift in their spectrum and observed the persistence of *Cooperia* and *Ostertagia* in some samples. We demonstrated that ITS-2 strongylid metabarcoding

from faecal samples without prior larval culture can detect GIN species composition even if the EPG is low and is a tool to monitor anthelmintic resistance.

Ethical statement

The research complied with the legal requirements of the Czech Republic and Romania. The samples were collected noninvasively, adhering to research protocols and Czech and Romania regulations.

Declaration of Competing Interest

All authors declare any financial and personal relationships with other people or organizations that could inappropriately influence (bias) their work.

Acknowledgements

This study was prepared as part of project QK1910204 funded by the Ministry of Agriculture of the Czech Republic. Work of the students was supported by Masaryk University (MUNI/A/1422/2022). We thank CF Genomics CEITEC MU, supported by the NCMG Research Infrastructure (LM2018132), for their support in obtaining the scientific data presented in this paper. Computational resources were provided by the "e-Infrastruktura CZ" project (e- INFRA CZ LM2018140) supported by the Ministry of Education, Youth and Sports of the Czech Republic. We would like to thank all breeders and veterinarians who participated in the study.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.vprsr.2023.100961.

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2025

Insights into infant strongyloidiasis, Papua New Guinea.

Emerg. Infect. Dis. 31, 1793–1801. https://doi.org/10.3201/eid3109.241923

Insights into Infant Strongyloidiasis, Papua New Guinea

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The human-infecting parasite *Strongyloides fuelleborni* subspecies *kellyi* has been reported from the island of New Guinea. We analyzed fecal DNA extracts (n = 164) from 19 infants in Papua New Guinea by using *Strongyloides* real-time PCR and undertook metabarcoding of *cox1* and 18S rRNA hypervariable regions I and IV loci. Eight infants were infected with *Strongyloides* spp.; 7 were infected with *S. fuelleborni* subsp. *fuelleborni* and 1 with a *Strongyloides* sp. previously misattributed to *S. fuelleborni* subsp. *kellyi*. Phylogenetic and haplotyping analyses indicated *S. fuelleborni* in Papua New Guinea belongs to the Indochina subclade of *S. fuelleborni* subsp. *fuelleborni* and is not a unique subspecies. We report molecular evidence of *S. fuelleborni* subsp. *fuelleborni* infection in humans in the Pacific. Our findings also demonstrate the potential co-existence of an undescribed human-infecting *Strongyloides* sp. on the island of New Guinea, indicating a need for renewed clinical and epidemiologic investigations into infant strongyloidiasis.

New Guinea, consisting of Indonesian New Guinea in the west and Papua New Guinea in the east, is the world's largest tropical island and a biodiversity hotspot. The island is home to the widespread and well-understood human pathogen *S. stercoralis* but also to a unique and enigmatic agent of human strongyloidiasis, the nematode *Strongyloides fuelleborni* subspecies *kellyi* (*1*–*3*). This *S. fuelleborni*–like intestinal nematode of humans was first reported in western Papua New Guinea in 1971 (*4*) and later found in Indonesian New Guinea (*5*).

There was taxonomic confusion over the identity of the New Guinea *Strongyloides* and its relationship to *S. fuelleborni* from Africa. Unlike *S. stercoralis*, which is passed as rhabditiform larvae, *S. fuelleborni* subsp. *kellyi* is passed as eggs, resembling those of larvated hookworms, in feces (*1*,*6*). Viney et al. observed the adult nematodes were morphologically distinguishable by subtle differences in the peri-vulval cuticle of parasitic female specimens and the phasmidial pore position of free-living male specimens (*3*). A separate isoenzyme electrophoresis analysis revealed that the 2 worm isolates clustered closely, separate from other mammal-infecting *Strongyloides* spp. (*7*). On the basis of those findings, subspeciation of *S. fuelleborni* into 2 subspecies was proposed (*3*): *S. fuelleborni* subsp. *fuelleborni*, and *S. fuelleborni* subsp. *kellyi* for the New Guinea nematode. Of note, strains of *S. fuelleborni* from Asia were not included in that analyses (*3*,*7*).

The epidemiology of *S. fuelleborni* subsp. *kellyi* nematodes is similarly enigmatic. Infection in children has been observed within 3 weeks after birth (*6*). One study found the incidence of infection rose rapidly in the first 12 months of life and then stabilized until 5 years of age, at which age-related incidence began to drop (*8*). How infection occurs in children so young is unclear. Transmammary transmission has been suggested (*6*), although a screening of breastmilk from 40 mothers in an endemic village during the 1970s revealed no larvae (*9*). Nonhuman primates, the animal reservoir of *S. fuelleborni* subsp. *fuelleborni*, are not native to New Guinea, and attempts to find an animal reservoir for *S. fuelleborni* subsp. *kellyi* by

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DOI: https://doi.org/10.3201/eid3109.241923

screening domestic animals, including pigs, chickens, and dogs from villages where infections occurred at high prevalence in humans, were unsuccessful (*4*,*7*).

Heavy *S. fuelleborni* subsp. *kellyi* infection has been implicated as the cause of swollen belly syndrome (SBS), a rapidly fatal disease in infants around 2 months of age (*6*). SBS is a protein-losing enteropathy characterized by eosinophilia, distended abdomen, diarrhea, and respiratory distress (*10*). The etiology of SBS remains poorly understood. SBS was observed during 1974–1983, predominantly in 2 remote villages in mountainous regions of Papua New Guinea, Kanabea (Gulf Province, ≈1270 m above sea level) and Wanuma (Madang Province, ≈750 m above sea level). Only occasional, sporadic cases were reported elsewhere (*6*). In Kanabea, infection with an egg-producing *Strongyloides* sp. was found in 100% of infants 3–8 weeks after birth, with a mean average egg burden of 94,000 eggs/mL of feces (*9*). SBS caused 8% of infant deaths in this village until specific anthelmintic therapy was introduced (*6*). This possible correlation of *Strongyloides* sp. infection to SBS is despite reports of high burdens and prevalence of *Strongyloides* eggs, presumed to be *S. fuelleborni* subsp. *kellyi*, in infants across several other regions of Papua New Guinea, where infections were associated with nutritional deficiencies but not SBS (*11*,*12*). An unknown factor was proposed to be involved in the development of SBS (*10*).

We found only 1 *S. fuelleborni* subsp. *kellyi* DNA sequence recorded to date (*13*), a 330-bp segment of 18S (small subunit) rRNA that covers the hypervariable region (HVR) I (*14*). Phylogenetic analysis on the basis of this region placed *S. fuelleborni* subsp. *kellyi* in a clade with *S. cebus*, *S. papillosus*, and *S. venezuelensis* but distant from *S. fuelleborni* subsp. *fuelleborni*. That work prompted calls to elevate *S. fuelleborni* subsp. *kellyi* to the species rank (*2*). However, a criticism of that finding is that 18S rRNA HVR-I is a poor marker for inferring taxonomic positions of *Strongyloides* spp (*15*,*16*). That gene region did not enable the separation of *Strongyloides* spp. with spiraled and straight ovary morphotypes, whereas nearly full-length 18S rRNA and 28S rRNA sequences did (*13*,*17*). Furthermore, 1 study (*13*) found *S. fuelleborni* subsp. *fuelleborni* to be phylogenetically closer to *S. stercoralis* than to *S. papillosus*, contradicting evidence suggested by mitochondrial genome data (*18*). Molecular taxonomy of *Strongyloides* spp. determined by using markers such as mitochondrial cytochrome oxidase subunit 1 (*cox*1) and 18S rRNA HVR-IV showed considerable consistency with whole-genome and mitochondrial genome analyses (*18*,*19*). The true phylogeny and taxonomic identity of *S. fuelleborni* subsp. *kellyi* could be elucidated by using those informative markers.

Despite its public health significance, *S. fuelleborni* subsp. *kellyi* remains a neglected and underexplored human helminth. This enigmatic causative agent of strongyloidiasis in New Guinea warrants focused research to clarify its identity and epidemiology. We analyzed fecal DNA extracts from 19 infants in Papua New Guinea to explore the identity of *S. fuelleborni* subsp. *kellyi* infection.

Methods

Sampling

We obtained samples from a 1-year longitudinal study investigating gut health in infants in Eastern Highlands Province, Papua New Guinea, during 2018–2019 (Figure 1). Ethics approval was obtained from the Papua New Guinea Institute of Medical Research Institutional Review Board (approval no. 1614) and the Papua New Guinea Medical Research Advisory Committee (approval no. 17.17). Infants were enrolled into the study within the first 5 weeks after birth. Initially, parents gave consent for participation in the study during antenatal clinic visits; after birth, informed consent was obtained from ≥1 parent or legal guardian. Fecal samples were collected from participants monthly for 12 months.

For our study, we investigated the presence of *Strongyloides* spp. in fecal samples from 19 total infants, 12 boys and 7 girls. For samples used in our study, infants were 0–101 days (average 30 days) of age when their first fecal sample was collected. Clinical data on the participants was limited, but no marked gastrointestinal pathology was noted during the study.

Fresh fecal samples were transported to the Papua New Guinea Institute of Medical Research (Goroka, Papua New Guinea), where they were stored at −70°C. Samples were subsequently sent to Queensland Institute of Medical Research-Berghofer Medical Research Institute (Herston, Queensland, Australia) and James Cook University (Townsville, Queensland, Australia) for laboratory analysis. We extracted DNA from each sample by using a QIAamp DNA mini kit (QIAGEN, https://www.qiagen.com). Before extraction, we mixed fecal samples (≈200 mg) with 500 μL of rapid 1-step extraction buffer, 15 μL of proteinase K, and 500 μL of 0.5-mm silica/zirconia beads (Daintree Scientific, http://www.daintreescientific.com.au) in 2-mL screw-cap tubes (Starstedt, https://www.sarstedt.com). We homogenized the tubes at 6,500 rpm for 40 seconds by using a Precellys

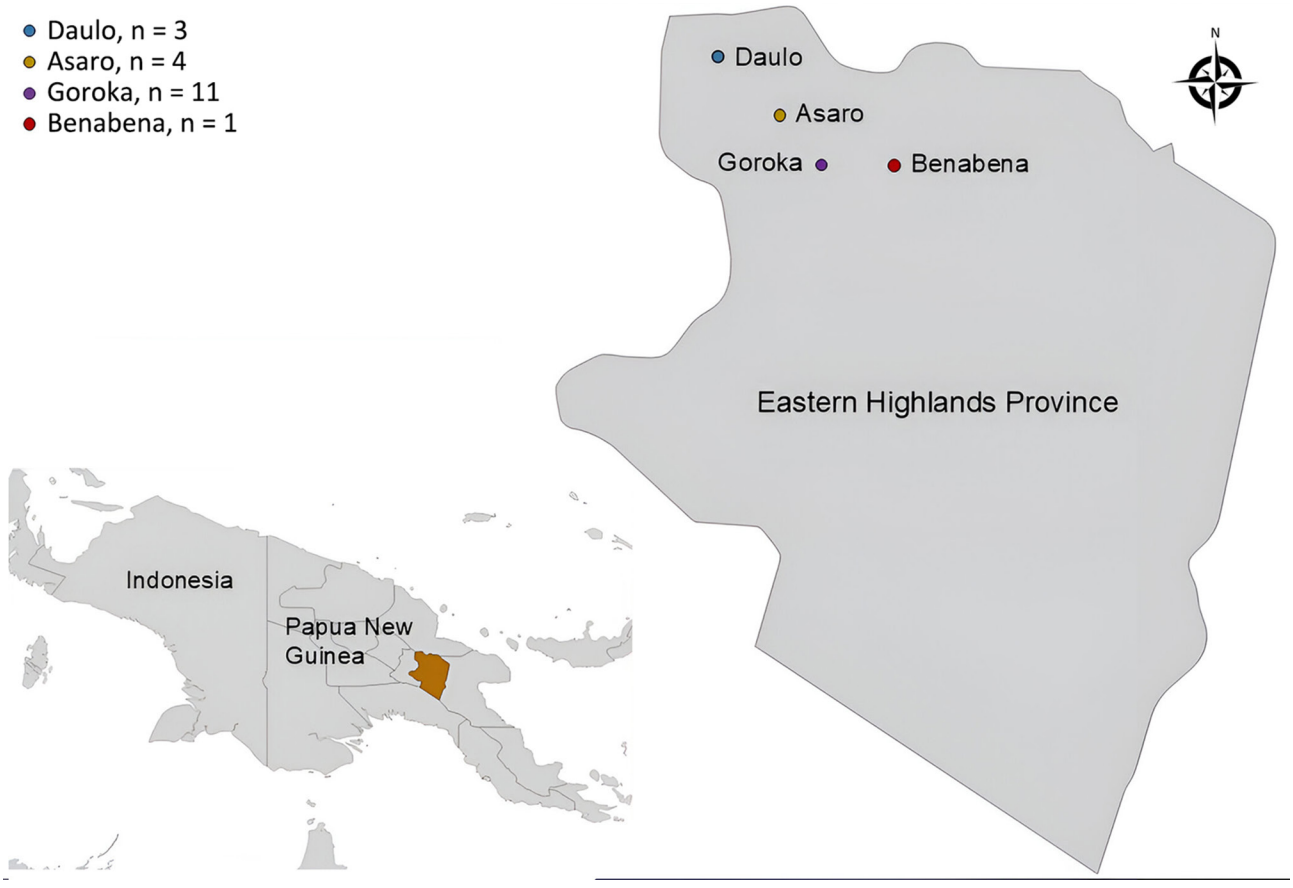


Figure 1. Sampling locations from study of infant strongyloidiasis, Eastern Highlands Province, Papua New Guinea. Participant numbers are shown for each location. Inset shows location of study area on the island of New Guinea.

homogenizer (Bertin Technologies, https://www.bertin-technologies.com). After homogenization, we incubated the samples at 95°C for 5 minutes and then centrifuged at 4,000 relative centrifugal force for 3 minutes. We transferred the supernatant to a new 1.5-mL tube and then followed the QIAGEN protocol for DNA extraction.

We assessed the extracted fecal DNA for quality and quantity by using a NanoDrop 2000 (Thermo Fisher Scientific, https://www.thermofisher.com). We used DNA samples with a concentration >10 ng/μL, and a 260/280 ratio between 1.6 and 2.10 as quantitative PCR templates. We reextracted 5 samples that did not meet those requirements; all subsequently reached the required DNA concentration. We then diluted the DNA 1:5 with Milli Q water (Thermo Fisher Scientific) and subjected it to 2 *Strongyloides* qPCRs (*20*,*21*). We confirmed positive qPCR results by triplicate testing and samples were considered positive when the cycle threshold (Ct) was <35. To tentatively infer the identity of the qPCR product, we conducted Sanger sequencing of positive amplicons from the 2 qPCRs, targeting a 471-bp region of 18S rRNA (*20*) and a 138-bp repeat sequence (*21*) by using the BigDye Direct Cycle Sequencing kit (Thermo Fisher Scientific).

We also performed a metabarcoding assay targeting 18S rRNA HVR-IV (≈252-bp) by using the Illumina MiSeq Reagent Nano Kit v2 (Illumina, https://www.illumina.com) (*16*). We then subjected positive samples to additional metabarcoding of the 18S rRNA HVR-I (≈432-bp) and partial *cox*1 (217-bp) genotyping targets (*16*) by using the same Illumina MiSeq platform. We conducted sequencing with 500 cycles (paired-end, 250-bp) for 18S rRNA products and 300 cycles (paired-end, 150-bp) for the *cox*1 products to ensure adequate sequencing coverage.

Statistical and Bioinformatic Analysis

We imported demographic and qPCR data into Excel (Microsoft, https://www.microsoft.com) for statistical analysis. We had the raw Illumina sequencing data analyzed by 3 blinded researchers at James Cook University and the Institute of Vertebrate Biology (IVB) through 2 different computational pipelines: a custom Geneious Prime (https://www.geneious.com)

workflow as previously published (*16*) at both James Cook University and IVB, and a combination of Skewer (*22*) and DADA2 (*23*) packages implemented in R software version 4.2.2 (The R Project for Statistical Computing, https://www.r-project.org) at IVB. Both pipelines incorporated read quality control, contig assembly, and haplotype assignment. We conducted maximum-likelihood phylogenetic analysis in MEGA 11 (https://www.megasoftware.net) and Bayesian inference phylogenetic analysis in MrBayes (https://nbisweden.github.io/MrBayes) on MUSCLE-aligned (https://github.com/rcedgar/muscle) *cox1* sequences by using the general time-reversible model for nucleotide substitutions. We then used Cohen's κ to assess the diagnostic agreement between different molecular methods for the detection of *Strongyloides* spp.

Results

A total of 164 fecal samples were collected from 19 infants during 5–13 occasions over the study period (Figure 1, 2). Eight (42%) of the 19 infants, comprising 6 boys and 2 girls, were found to be infected with a *Strongyloides* spp. Infections were detected at an average age of 212 days (range 94–334 days) (Figure 2). Of the 8 positive cases, fecal metabarcoding at *cox1* and 18S rRNA HVR-I and HVR-IV loci identified 6 infections as *S. fuelleborni* subsp. *fuelleborni* and 1 as a *Strongyloides* sp. previously attributed to *S. fuelleborni* subsp. *kellyi* (*13*) (Table). The remaining positive sample could only be amplified at the qPCR 18S rRNA target. That 471-bp sequence was 99.2% identical to a published *S. fuelleborni* subsp. *fuelleborni* sequence (GenBank accession no. AB272235) by BLAST analysis (https://blast.ncbi.nlm.nih.gov/).

The previously published genus-specific qPCR method (*20*) performed comparably with a previously published 18S rRNA HVR-IV metabarcoding method (*16*) in detecting *Strongyloides* infections, yielding a Cohen's κ coefficient of 0.85. The second qPCR method (*21*) did not detect *S. fuelleborni* subsp. *fuelleborni* but did detect the *Strongyloides* spp. previously considered *S. fuelleborni* subsp. *kellyi* (Figure 3).

We obtained sequences of 18S rRNA HVR-I (432-bp) and HVR-IV (≈252-bp) from 7 samples (Table). Six positive samples harbored HVR-IV haplotype S and HVR-I haplotype XIV, both genotypes previously identified in all *S. fuelleborni* subsp. *fuelleborni* isolates from Asia. The remaining positive sample was infected with 3 HVR-I haplotypes (deposited into GenBank under accession nos. PV489780–2): two with 275-bp sequences identical to the only published sequence of *S. fuelleborni* subsp. *kellyi* (GenBank accession no. AJ417029) (*13*) and the third differing by 1 single-nucleotide polymorphism (SNP) (T-C at position 28). HVR-I sequences of that *Strongyloides* sp. (432-bp) differed from those of *S. ransomi* (GenBank accession nos. LC324901, OP288111) by 2 SNPs and from *S. venezuelensis* (GenBank accession no. AB923887) by 1 SNP (Figure 4). At the HVR-IV locus, the 248-bp sequence was 100% identical to sequences of *S. ransomi* (GenBank accession nos. OP288111, KU724127) and *S. venezuelensis* (GenBank accession no. AB923887).

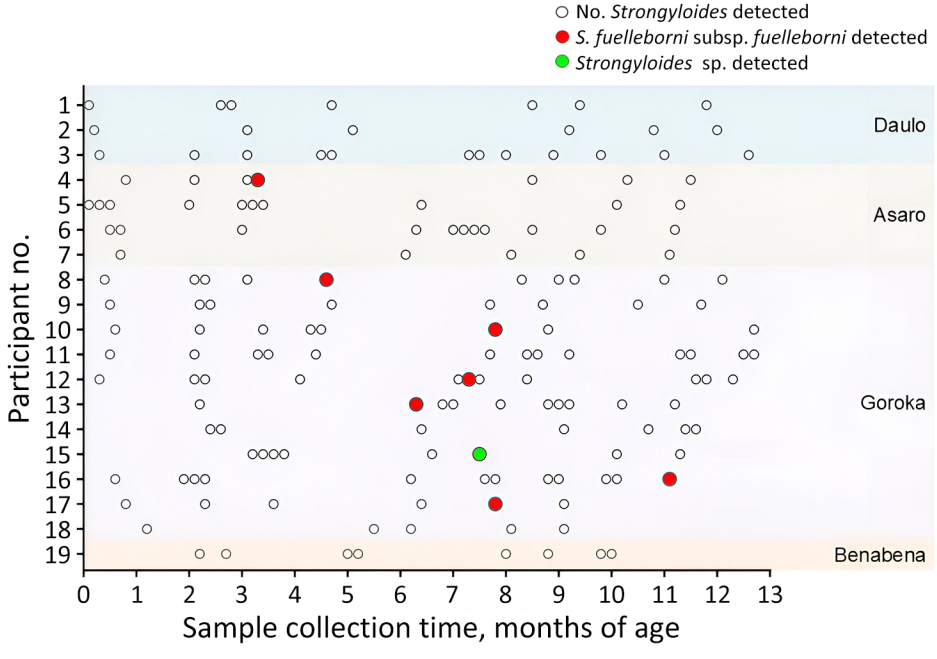


Figure 2. Sampling frequency and *Strongyloides* infection in infants from Eastern Highlands Province, Papua New Guinea. At least 5 samples were collected from each infant enrolled during the first 13 months of life. Samples that demonstrated molecular evidence of *Strongyloides* spp. infection are shown. Sample locations are indicated at right.

Table. *Strongyloides* samples analyzed and their genotypes in study of infant strongyloidiasis, Papua New Guinea*

Sample no.	Patient age, d/sex	Species detected	18S rRNA HVR-IV haplotypes	18S rRNA HVR-I haplotypes	*cox*1 haplotypes
1	205/M	*S. fuelleborni* subsp. *fuelleborni*	S (PQ774622)	XIV (PV489783)	3 OTUs (PQ774615–7)
2	94/M	*S. fuelleborni* subsp. *fuelleborni*	S (PQ774622)	XIV (PV489783)	3 OTUs (PQ774615–7)
3	140/F	*S. fuelleborni* subsp. *fuelleborni*	S (PQ774622)	XIV (PV489783)	2 OTUs (PQ774615, PQ774616)
4	234/M	*S. fuelleborni* subsp. *fuelleborni*	S (PQ774622)	XIV (PV489783)	2 OTUs (PQ774615, PQ774616)
5	227/F	*Strongyloides* spp.	1 OTU (PQ774624)	3 OTUs (PV489780–2)	NA†
6	225/M	*S. fuelleborni* subsp. *fuelleborni*	S (PQ774622)	XIV (PV489783)	2 OTUs (PQ774616, PQ774617)
7	236/M	*S. fuelleborni* subsp. *fuelleborni*	S (PQ774622)	XIV (PV489783)	2 OTUs (PQ774615, PQ774616)
8	334/M	*S. fuelleborni* subsp. *fuelleborni*	NA‡	NA‡	NA‡

*Values in parentheses are GenBank accession numbers. HVR, hypervariable region; NA, not available; OTU, operational taxonomic unit.
†*cox1* region cPCR did not amplify.
‡Metabarcoding on the 18S rRNA HVR-IV locus yielded no detectable product on agar electrophoresis.

cox1 sequences were available for 6 samples, all assigned to *S. fuelleborni* subsp. *fuelleborni* (Table). The seventh sample, containing the genetically distinct *Strongyloides* sp., did not amplify. We identified 3 separate *S. fuelleborni* subsp. *fuelleborni* haplotypes (deposited into GenBank accession nos. PQ774615–7). Maximum-likelihood and Bayesian inference phylogenetic analyses on the *cox1* locus placed *S. fuelleborni* subsp. *fuelleborni* from Papua New Guinea in a clade with *S. fuelleborni* subsp. *fuelleborni* from Myanmar *rhesus macaques* (GenBank accession no. OL672153). Those sequences also clustered closely with *S. fuelleborni* subsp. *fuelleborni* from Bangladesh (GenBank accession nos. OR805176 and OR805181) (Figure 5).

Discussion

We provide molecular evidence of human infections with *S. fuelleborni* subsp. *fuelleborni* outside of Asia and Africa. The potential existence of an undescribed human-infecting *Strongyloides* sp., previously misattributed to *S. fuelleborni* subsp. *kellyi* (*13*), is also suggested. On the basis of those findings, we hypothesize that ≥2 genetically distinct, non–*S. stercoralis Strongyloides* nematodes infect humans in Papua New Guinea and that *S. fuelleborni* subsp. *kellyi*, as previously described (*3*), is not a unique subspecies but is likely synonymous with the Asia-Pacific clade of *S. fuelleborni* subsp. *fuelleborni*.

Our phylogenetic findings corroborate a previously published study (*3*) that described very subtle morphologic distinctions between *S. fuelleborni* adult isolates from Africa and Papua New Guinea. Both strains differed markedly in morphology from *S. ransomi* in Papua New Guinea pigs. Because those analyses did not include isolates of *S. fuelleborni* subsp. *fuelleborni* from Asia, and no morphologic studies of this nematode from Asia exist, it is possible that study described the first representatives of what is now recognized as the clade of *S. fuelleborni* subsp. *fuelleborni* from Asia (*3*). Further comparative morphologic and genomic analyses of adult isolates from Africa, Asia, and the Pacific are required to test that hypothesis. If confirmed, the finding would warrant a revision of current subspecies epithets to reflect the divergence between Africa and Asia-Pacific *S. fuelleborni*.

In Africa and Asia, *S. fuelleborni* subsp. *fuelleborni* is a common infection of nonhuman primates and is considered a zoonosis originating from those animals (*24*,*25*). Multiple studies suggest human-to-human transmission of *S. fuelleborni* subsp. *fuelleborni* occurs in some regions of Africa (*25*,*26*). This suggestion is supported by genetic analysis (*27*) identifying a human-specific subpopulation among *S. fuelleborni* subsp. *fuelleborni* isolates from Africa. Because of the absence of a nonhuman primate reservoir on the island of New Guinea, it is likely that *S. fuelleborni* subsp. *fuelleborni* has adapted to exclusive human-to-human

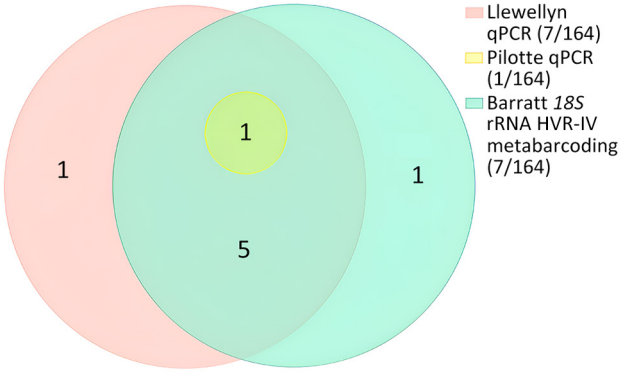


Figure 3. Euler diagram showing the performance of 2 qPCRs (*20*,*21*) and 18S rRNA HVR-IV metabarcoding (*16*) for the detection of *Strongyloides* spp. in 164 infant fecal samples from Papua New Guinea. Values in parentheses are no. positive samples/total no. tested. HVR, hypervariable region; qPCR, quantitative PCR.

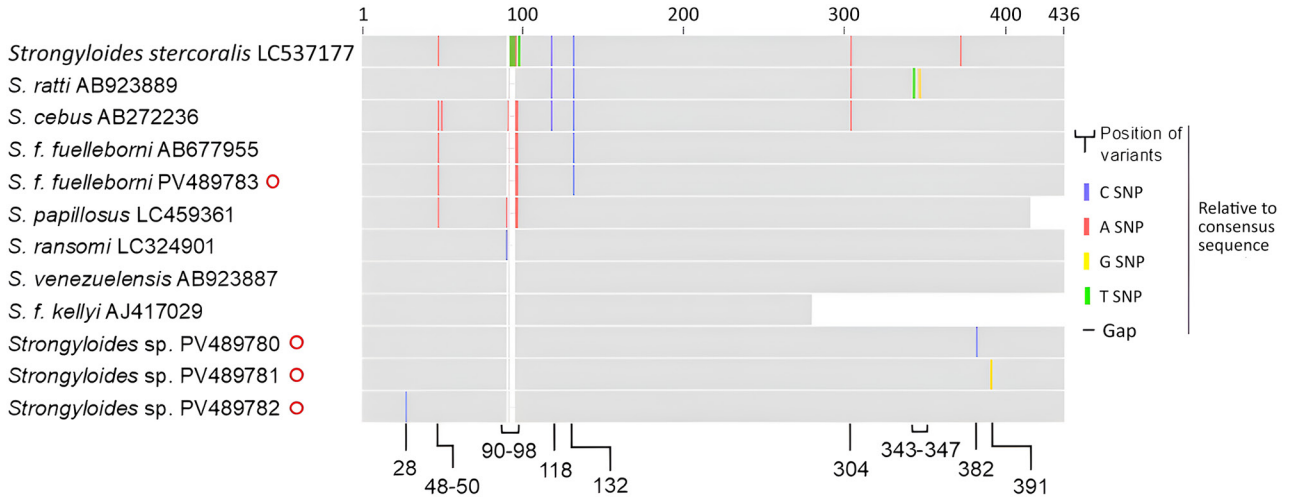


Figure 4. Schematic of MUSCLE-aligned (https://github.com/rcedgar/muscle) *Strongyloides* sp. *18S rRNA* HVR-I sequences from study on infant strongyloidiasis, Papua New Guinea. Sequences obtained are marked with red circles. Previously published sequences are annotated with their GenBank accession numbers. *S. f. fuelleborni*, *S. fuelleborni* subsp. *fuelleborni*; *S. f. kellyi*, *S. fuelleborni* subsp. *kellyi*.

transmission after being introduced to Papua New Guinea through human migration (*28*).

The distinct *Strongyloides* genospecies identified in 1 infant from this study corresponds to the genospecies previously misattributed to *S. fuelleborni subsp. kellyi* (*13*). This species was indistinguishable from *S. ransomi* and *S. venezuelensis* at the 18S rRNA HVR-IV locus and exhibited only 1–2 SNPs at the HVR-I locus, suggesting a recent common ancestry among them. An earlier isoenzyme electrophoretic analysis (*7*) found that 4 of 26 *Strongyloides* isolates recovered from persons in Papua New Guinea clustered closely with *S. ransomi* from local pigs. Although there was speculation those samples might have originated from pigs, it is plausible they represented the same distinct species identified in this and a previous study (*13*). However, caution is warranted when interpreting taxonomic placements based solely on single-locus 18S rRNA data from fecal DNA, without accompanying morphologic data, because those inferences might be artifactual. Detailed morphologic analysis of adult isolates, combined with whole-genome or mitochondrial genome sequencing, is needed to resolve the taxonomic status of this nematode.

Historical reports of *S. fuelleborni* subsp. *kellyi* predated the molecular era and relied solely on microscopic identification of *Strongyloides* eggs (*4*,*6*,*8*,*9*,*12*,*28*). Those data require reassessment because they might conflate infections with 2 co-endemic human-infecting *Strongyloides* spp. nematodes. Surveys conducted during 1976–1997 in Papua New Guinea reported *S. fuelleborni* subsp. *kellyi* prevalence of 20%–93% in children, with rates reaching 60% within the first year of life (*6*,*8*,*9*,*12*,*28*). We similarly found a high incidence of *S. fuelleborni* infection (7/19), but also 1 infection (1/19) with an undescribed *Strongyloides* sp. in Papua New Guinea infants. Future surveillance for strongyloidiasis in New Guinea should use species-specific molecular tools to differentiate those 2 agents.

Our findings provide explanation for much of the unknown epidemiology of infant strongyloidiasis in Papua New Guinea. Patent *S. fuelleborni* infections in infants as young as 18 days of age have been found in Papua New Guinea (*6*). Sampling of breastmilk from mothers in Papua New Guinea (*29*) did not identify any *Strongyloides* larvae; however, difficulties in obtaining fecal samples from those mothers left their infection status uncertain. In the same village, the prevalence of *Strongyloides* spp. in adult feces was only 14% (*12*). In a survey of 25 lactating mothers of infants with confirmed *S. fuelleborni* subsp. *fuelleborni* infection in the Democratic Republic of the Congo, *Strongyloides* spp. filariform larvae were found in the breast milk of 1 mother (*30*). That finding suggests transmammary transmission could be responsible for the high rate of infection in infants as young as 50–74 days of age in that region (*30*). Because our genetic analysis indicates the worms we identified belong to clades of the same species, it is plausible that transmammary transmission to Papua New Guinea infants might occur. That speculation warrants further investigation, and the use of molecular genotyping tools might be necessary to track transmission patterns.

The attribution of *S. fuelleborni* subsp. *kellyi* as the causative agent of infantile SBS requires further validation. Despite *S. fuelleborni* infection being widespread

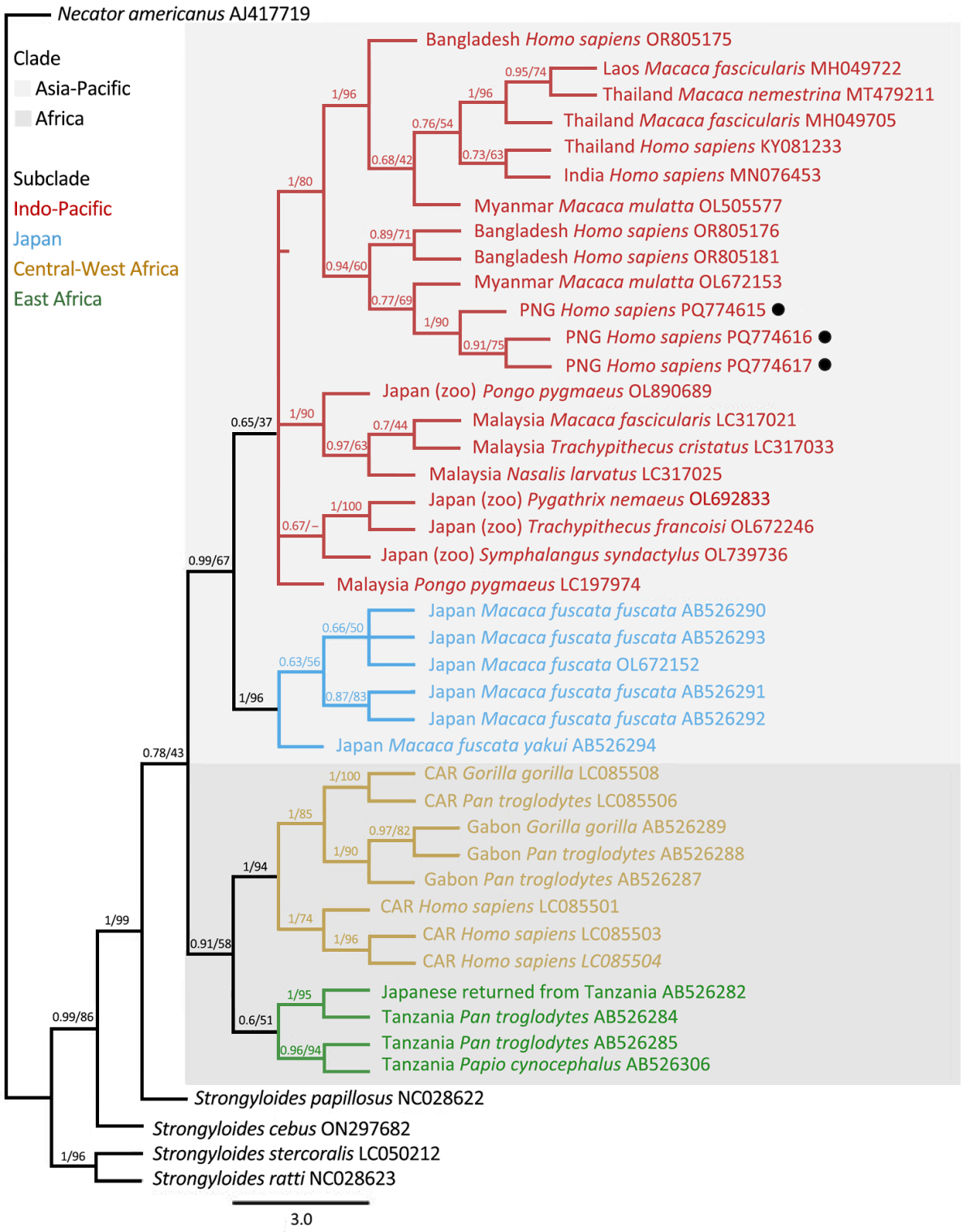


Figure 5. Phylogenetic tree of *Strongyloides fuelleborni* subsp. *fuelleborni* on the basis of *cox*1 sequences from study on infant strongyloidiasis in Papua New Guinea. Bayesian posterior probability and maximum-likelihood bootstrap support percentages (1,000 replicates) are indicated at the nodes. Black dots indicate sequences obtained in this study. Previously published sequences are annotated with their country of origin, host species, and GenBank accession numbers. Clades and subclades of *S. fuelleborni* subsp. *fuelleborni* are color-coded on the basis of geographic regions. Scale bar indicates number of substitutions per site. –, no statistics are available; CAR, Central African Republic; PNG, Papua New Guinea

in children in some parts of Africa, SBS has not been reported from that continent (*25*). Neither has SBS associated with *S. fuelleborni* infection been documented in Asia. The detection of a *Strongyloides* genospecies closely related to *S. ransomi* of pigs raises an alternative hypothesis regarding the etiology of SBS in Papua New Guinea. In newborn suckling piglets, *S. ransomi* infection causes a protein-losing enteropathy characterized by villus atrophy, malabsorption, diarrhea, progressive dehydration, hypoproteinaemia, anemia, anorexia, emaciation, sudden death (*31*,*32*), and reduced hepatic protein synthesis (*33*), a clinical picture strikingly similar to SBS in infants from Papua New Guinea (*1*,*6*). Because of the substantial genetic similarity observed between *S. ransomi* and this genospecies, a shared pathogenic mechanism is plausible. *S. ransomi* detection in only 1 of 19 infants also mirrors the rare and sporadic occurrence of SBS previously reported in this population (*6*,*9*,*10*). Further research is needed to explore this hypothesis and clarify the etiology and epidemiology of SBS.

In our study, not all diagnostic *Strongyloides* spp. qPCRs detected *S. fuelleborni* infection. The Llewelyn modification (*20*) of Verweij (*34*) qPCR appears generic and detected all except 1 *S. fuelleborni* infection. In contrast, the Pilotte *S. stercoralis* qPCR (*21*) only amplified DNA of the undescribed *Strongyloides* sp. and did not detect *S. fuelleborni* infections. That finding demonstrates that the specificity of PCR diagnostics used must be considered in future surveillance of *Strongyloides* spp. infections in humans and animals, because the choice of qPCR might markedly affect the findings of any survey.

In summary, we present molecular evidence of human infections with *S. fuelleborni* subsp. *fuelleborni* nematodes in Papua New Guinea. On the basis of this evidence and existing morphologic data on *S. fuelleborni* subsp. *kellyi*, we hypothesize that *S. fuelleborni* subsp. *kellyi* is not a unique subspecies but rather represents *S. fuelleborni* subsp. *fuelleborni* Asia-Pacific clade infections occurring in humans in New Guinea. We further molecularly identified an undescribed *Strongyloides* species in 1 infant from Papua New Guinea and raise questions about the possible role of this undescribed species in the etiology of infantile SBS. Renewed clinical, epidemiologic, and taxonomic investigations into infant strongyloidiasis in this region are needed to increase clinician awareness of such infections and guide prevention and treatment efforts.

Acknowledgments

We thank the participants and their families for providing stool samples. We thank the leadership and staff at Papua New Guinea Institute of Medical Research, particularly members of the Infection and Immunity Unit, for their collaboration on this project.

This work was partially funded by the National Health and Medical Research Council (investigator grant no. APP1194462). H.Z. and J.H. receive an Australian Government Research Training Program scholarship from James Cook University, Australia.

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Ms. Zhao is a PhD candidate at James Cook University. Her research focuses on the molecular taxonomy and epidemiology of *Strongyloides* species in humans and companion animals.

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2024a

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Parasitology 151, 587–593. https://doi.org/10.1017/S0031182024000568

Parasitology

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High-throughput sequencing of *Strongyloides stercoralis* – a fatal disseminated infection in a dog

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Research Article

Cite this article: Nosková E, Svobodová V, Hypská V, Cerezo-Echevarria A, Kurucová T, Ilík V, Modrý D, Pafčo B (2024). High-throughput sequencing of *Strongyloides stercoralis* – a fatal disseminated infection in a dog. *Parasitology* **151**, 587–593. https://doi.org/10.1017/S0031182024000568

Received: 17 February 2024
Revised: 26 April 2024
Accepted: 28 April 2024
First published online: 8 May 2024

Keywords:
dog; high-throughput sequencing; *Strongyloides stercoralis*; Strongyloidiasis; zoonotic

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Abstract

The rhabditid nematode *Strongyloides stercoralis* is known worldwide as the causative agent of strongyloidiasis in humans. In addition to public health concerns, *S. stercoralis* also infects dogs, which represent a possible reservoir for potentially zoonotic transmissions. We describe the first confirmed case of fatal disseminated infection in a dog in the Czech Republic. The microscopic and histological results were supported by a complex genotyping approach. Using high-throughput sequencing of the hypervariable region (HVR-IV) of 18S rDNA and Sanger sequencing of the partial cytochrome c oxidase subunit 1 gene (*cox1*), the potentially zoonotic haplotype/lineage A of *S. stercoralis* was confirmed, while the solely canine haplotype/lineage B was not found. The development of the disease is mainly associated with immunodeficiency, and in this case, it was triggered by inappropriate treatment, in particular the use of corticosteroids.

Introduction

Strongyloides stercoralis infects a range of hosts, including canids, felids, humans and non-human primates (NHPs). Resulting infection, usually termed strongyloidiasis, remains a major veterinary and public health challenge globally (Bradbury *et al.*, 2021). The parasite has a unique life cycle in which it alternates between free-living and parasitic stages. The free-living stage is a single generation with a short life span. The parasitic generations include only parthenogenetic females living in the small intestine, that are ovoviviparous. The autoinfective filariform larvae may develop from rhabditiform larvae already in the intestine and can invade the intestinal mucosa or perianal area, causing autoinfection (Sandground, 1925; Streit, 2008; Page *et al.*, 2018). In dogs, the infection may be asymptomatic. However, respiratory, dermatologic and gastrointestinal signs accompanied by vomiting and anorexia may occur in young animals, especially in puppies (Robertson and Thompson, 2002; Paradies *et al.*, 2017), sometimes with fatal consequences (Dillard *et al.*, 2007; Unterköfler *et al.*, 2022). So far, none of the available anthelmintics are formally approved for the treatment of strongyloidiasis in dogs. In a few cases, successful off-label treatment has been demonstrated using moxidectin, milbemycin, praziquantel, fenbendazole and ivermectin, often in combination (Eydal and Skírnisson, 2016; Unterköfler *et al.*, 2022; Deak *et al.*, 2023). However, a single dose is not sufficient to eradicate the parasite, and successful therapy requires repeated applications (Itoh *et al.*, 2009; Paradies *et al.*, 2017; Basso *et al.*, 2019). On the other hand, the administration of some drugs, such as glucocorticoids, can lead to a hyperinfection and worsen the health status (Basso *et al.*, 2019), as they immunosuppress the host and act directly on the larvae, promoting accelerated moulting to the autoinfective stage (Ramanathan and Nutman, 2008).

Strongyloides stercoralis is being intensively studied in dogs because of its zoonotic potential (Gorgani-Firouzjaee *et al.*, 2022). Infected free-roaming dogs could play a role in spreading the infection to other hosts including humans (Jaleta *et al.*, 2017; Nagayasu *et al.*, 2017) and reciprocally, humans could be a possible source of infection for dogs living in close contact with them, but this is yet to be proven (Bradbury and Streit, 2024). This extremely close coexistence between domestic carnivores and humans has created conditions for the mutual transmission of pathogenic and non-pathogenic organisms and has become part of the One Health concept, especially in Western society (Gray and Young, 2011; van Knapen and Overgaauw, 2014; Wetzels *et al.*, 2021).

In the case of *S. stercoralis* two main haplotypes/lineages have been described: the potentially zoonotic haplotype/lineage A, which occurs in humans, dogs and mainly captive NHPs

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and the likely solely canine haplotype/lineage B (Bradbury *et al.*, 2021). While haplotype/lineage A appears to be more widespread in dogs worldwide, haplotype/lineage B has been detected only in Australia, Cambodia, Myanmar and Grenada (Nosková *et al.*, 2024). However, numerous cases of *S. stercoralis* infections in dogs are reported without genetic analysis (Schnyder *et al.*, 2022), so the exact distribution of individual haplotypes/lineages worldwide is most likely inaccurate.

Basic microscopy, ideally following the Baermann larvoscopy (Schnyder *et al.*, 2022), is essential for the detection of first-stage *S. stercoralis* larvae (L1; or occasionally infective L3 and parasitic females) from faecal samples (Basso *et al.*, 2019; Schnyder *et al.*, 2022), while oval eggs with U-shaped larvae are typical for some other *Strongyloides* species (Viney and Lok, 2015). Genetic-based methods are essential for the resolution of the individual haplotypes/lineages. Both the hypervariable region (HVR-IV) of 18S rDNA and cytochrome c oxidase subunit I (*cox1*) have the discriminatory power to differentiate *S. stercoralis* haplotypes/lineages (Beknazarova *et al.*, 2019) and should be selected as the currently most appropriate markers, as there are no other discriminatory methods. Some haplotypes were recognized using the HVR-IV region of 18S, which correspond to the particular *cox1* lineage, indicating that mixed infections with other *Strongyloides* species are not uncommon (Barratt *et al.*, 2019) and this could be true also for the haplotypes/lineages of *S. stercoralis* in dogs. In recent years, increasingly complex genotyping methods have been used in *Strongyloides* research that enable the detection of multi-haplotypes infections in faecal samples (Barratt *et al.*, 2019).

After receiving a sample from a dog with disseminated *Strongyloides* infection, we aimed to uncover which genetic haplotype and lineage is the causative agent of the disease. A high-throughput sequencing (HTS) approach was used to determine whether multiple haplotypes of HVR-IV overlap during this fatal infection. Sanger sequencing complemented the HTS results on the *cox1* and HVR-I regions.

Material and methods

Clinical case

A one-year-old crossbred dog female was adopted from a Slovakian shelter in April 2022 with no medical records. From the beginning, the dog had repeated diarrhoea. Before the case was submitted to us, coproscopic examination was either not performed or included only faecal flotation with a negative result. In September 2022, the dog was presented to a veterinary clinic in Hradec Králové, Czech Republic as subfebrile, with profuse diarrhoea, apathy, cachexia (BCS 1/5). A fresh faecal sample was collected and examined using Sheather's flotation at the clinic. Baermann larvoscopy was not performed. Infrequent damaged unspecified larvae were microscopically detected after Sheather's flotation. The hematological examination was unremarkable. In the biochemical profile, liver enzymes and bilirubin were slightly elevated, amylase and pancreatic lipase were markedly elevated together with mild hyponatremia. The dog was dewormed with a single dose of moxidectin combined with imidacloprid (Advocate spot on). Furthermore, amoxicillin/clavulanate (22 mg kg^{-1}, Noroclav inject.) and marbofloxacin (3 mg kg^{-1}, Marbocyl FD inject.) were administrated. No larvae were detected after 6 days during the control Sheather's flotation. Dog condition did not improve; loss of appetite and vomiting persisted. Radiographic examination of the chest in latero-lateral projection showed a slightly accentuated bronchial pattern; endoscopy revealed abnormal findings in the duodenum, which was markedly hyperaemic throughout and had numerous miliary coagulum. The dog's condition deteriorated, with opisthotonus and bloody, profuse diarrhoea. Therefore, endoscopic examination, biopsy and subsequent histological examination were performed (LABOKLIN GmbH & Co. KG, see Histologic examination below). Based on the histological results, a faecal sample was sent to the University of Veterinary Sciences Brno for coproscopic examination. *Strongyloides* infection was diagnosed based on the microscopic and molecular identification and treated with Flubendazole (Flubenol KH paste) 22 mg kg^{-1} for 3 consecutive days. During the control flotation of the sample from the 3rd day after flubendazole administration (19th day after Advocate spot application), non-motile *Stongyloides* stages were observed. On the fourth day after initiation of anthelmintic therapy, the dog developed severe respiratory distress due to polypnea associated with complete anorexia. The respiratory distress was treated with corticosteroids, methylprednisolone (1 mg kg^{-1}, SoluMedrol 40 mg inject.) and opiates (butorphanol 0.2 mg kg^{-1}, Butomidor inject.), which led to relief of the polypnea. The dog, however, died the following day.

Histologic examination

A total of 27 samples, taken during the endoscopy, from the stomach, duodenum, ileum and colon were submitted to the LABOKLIN GmbH and Co. KG, for histological examination. The samples ranged from 1 mm in diameter up to 5 × 2 × 2 mm and included mostly mucosal surfaces. All samples were fixed in 10% phosphate-buffered formalin for 24–48 h to ensure correct fixation. They were then trimmed and processed according to laboratory standards (Fischer *et al.*, 2008), embedded in paraffin wax, cut at 4–5 µm thickness and stained with Haematoxylin-Eosin (HE) and Periodic Acid-Schiff stain (PAS). The slides were scanned and analysed using image analysis software (NIS-elements software) (Nikon, Japan); Aperio ImageScope (Leica, Germany).

Faecal sample preparation, DNA extraction and molecular assays

A 200 ml suspension of faecal sample in water for molecular identification and the same amount of suspension fixed in formalin for coproscopic examination were sent to Department of Pathology and Parasitology, University of Veterinary Sciences Brno, Czech Republic in October 2022. Due to submission of a non-standard sample, the Baermann larvoscopy was not performed. The nematode stages in formalin-preserved sample were let to sediment, collected individually by a pipette and observed and measured using the light microscope with Nomarski differential contrast (Olympus BX41) at 100 × magnification and photographed (Olympus AX70).

The faecal–water suspension was centrifuged in 50-ml falcon tubes. The sediment was transferred into 1.5 ml tubes and dried overnight at 37°C before DNA isolation (Murphy *et al.*, 2000). Total DNA from one sample was extracted using the DNeasy PowerSoil Kit (QIAGEN, Germany) according to the manufacturer's protocol. The most frequently used markers for *Strongyloides* spp. amplification were selected (Barratt *et al.*, 2019), namely two hypervariable regions (HVRs) of 18S rDNA (HVR-I and HVR-IV) and the mitochondrial gene *cox1*. The HVR-I region (434 bp) was amplified using primers New_HVR-I_F and New_HVR-I_R, whereas *cox1* (650 bp) was amplified with pair primers TJ5207 and TJ5208 (Jaleta *et al.*, 2017). The volume and mixture of the PCR reaction of HVR-I region and *cox1* were performed according to Nosková *et al.* (2023) (Table 1). Sanger sequencing was performed by Macrogen Europe (Netherlands). To reveal the potential occurrence of multiple *Strongyloides* haplotypes, the library for HTS was created using a 2-step-PCR approach, performed using primers New_HVR-IV_F and New_HVR-IV_R

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Table 1. Primers and reaction conditions

Primers	Sequence 5′→ 3′	Reaction conditions	Region of gene	References
TJ5207 TJ5208	TTTGATTGTTACCTGCTTCTATTTT TTTTACACCAGTAGGAACAGCAA	2 min at 94°C; 35 cycles of 20 s at 94°C, 15 s at 50°C, 90 s at 72°C; and 7 min at 72°C	*cox1*	Jaleta *et al.* (2017)
HVRI F HVRI R	GCTCATTATAACAGCTATAGACTACACGGTA CCACAACAATCATTTTATGCACTTGG	3 min at 95°C; 35 cycles of 15 s at 95°C, 15 s at 60°C, 15 s at 72°Cs; and 1 min at 72°C	18S rDNA (HVR-I)	Barratt *et al.* (2019)
HVRIV F HVRIV R	CGGGCCGGACACTATAAGG ATCTCTAAACAGGAACATAATGATCACTAC	3 min at 95°C; 30 cycles of 15 s at 95°C, 15 s at 60°C, 15 s at 72°Cs; and 1 min at 72°C	18S rDNA (HVR-IV)	
Nextera adaptor F	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG	3 min at 95°C; 16 cycles of 15 s at 95°C, 30 s at 55°C, 30 s at 72°C; and 72°C for 3 min	-	16S metagenomic sequencing library preparation protocol, Illumina
Nextera adaptor R	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG			

(Barratt *et al.*, 2019), amplifying the HVR-IV region (255 bp). In the first step of PCR inner locus specific primers for the HVR-IV region amplification (New_HVR-IV_F and New_HVR-IV_R) were used, additionally containing nextera adaptor sequences at 5′ ends following design from a 16S Metagenomic Sequencing Library Preparation protocol. The first amplification was performed in a 10 µL reaction volume, including Kapa 2 G Robust Hot Star polymerase (Kapa Biosystems, United States), 0.25 µL of each primer and 2 μL of DNA template, before the second amplification was performed with the same polymerase, 4 µL of Nextera primers with adaptors and 2 µL diluted product in a 20 µL reaction volume (Table 1). The final library was sequenced using MiSeq Reagent Kit v3 (2 × 300 bp pair-end reads) by Illumina MiSeq platform (Illumina, USA).

Data processing and phylogenetic analyses

Raw FASTQ sequences of the HVR-I of 18S rDNA and partial *cox1* genes obtained after Sanger sequencing were checked manually and trimmed in Geneious 9.1.5 (www.geneious.com). Raw HVR-IV of 18S rDNA FASTQ sequences obtained using HTS were trimmed using Skewer (Jiang *et al.*, 2014) and denoised using the dada2 package (Callahan *et al.*, 2016) in Rstudio (https://www.rstudio.com). The taxonomic identification for all sequences (one from HVR-I, one from HVR-IV and one from *cox1*) were performed using BLAST software against the BLAST nt database (Altschul *et al.*, 1990), available online at the National Center for Biotechnology Information, https://www.ncbi.nlm.nih.gov/.

For phylogenetic analyses, 154 sequences of the partial *cox1* gene of *S. stercoralis* from dogs were downloaded from Genbank and checked for duplicates. In addition, 12 sequences from humans and NHPs were included. Phylogenetic analysis of partial *cox1* sequences was reconstructed by Bayesian inference (BI) in MrBayes 3.2.6 (Huelsenbeck and Ronquist, 2001). Only unique dog sequences from 11 localities were included in the phylogenetic analysis. The Muscle alignment (715 bp) of *cox1* consisted of one sequence obtained during the present project and 61 sequences from Genbank, including *Necator americanus* (AJ417719) as an out-group. Nodal support was assessed to 10^6 replicates. Substitution GTR + G model was used as selected by Modelgenerator (Keane *et al.*, 2006).

Results

Detection of adult parthenogenetic female and pathological findings

Within the intestinal samples of duodenum, ileum and colon, the mucosa, lamina propria and submucosa, when available, were diffusely infiltrated and expanded by moderate numbers of lymphocytes, cells, as well as scattered eosinophils. Embedded within the intestinal crypts there were numerous cross and tangential sections of degenerated adult nematodes and larvae (Fig. 1A). The villi were overall slightly blunted and occasionally fused. The intestinal crypts often contained an increased amount of mucinoid material, as well as a small number of cellular debris. The associated lymphoid tissue in these areas was often prominent and hyperplastic. The stomach samples were histologically unremarkable, with no evidence of parasites or overt inflammation, other than mild superficial erosion and apical haemorrhages. High number of adult parthenogenetic *S. stercoralis* females (average length 1300 µm) were observed in faecal suspension using microscopy. The typical morphological characters such as elongated (filariform) oesophagus (Fig. 1B), vulval region (Fig. 1C), buccal cavity (Fig. 1D) and tail (Fig. 1E) were observed.

Haplotypes and phylogenetic analysis of S. stercoralis

Strongyloides stercoralis sequences of the HVR-I (466 bp) and HVR-IV (290 bp) of 18S rDNA and partial *cox1* (628 bp) were obtained from the examined dog faecal sample. BLAST search showed that sequence of the HVR-I region corresponded with haplotype VI while HVR-IV corresponded with haplotype A as marked in Barratt *et al.* (2019). Moreover, 97 046 high-quality reads were obtained using HTS and only potentially zoonotic HVR-IV haplotype A was detected, while the solely canine haplotype B was not recorded. The *cox1* results also detected the potentially zoonotic lineage A. The resulting BI tree based on *cox1* showed two separate *S. stercoralis* clades corresponding to lineages A and B, with our *cox1* sequence (OR587862) placed in the A lineage (Fig. 2), clustering with high support (0.84 BI) into a subclade comprising two isolates from Japanese dogs and one isolate from a dog from USA. The pairwise sequence distance (PSD) within this sub-clade did not reach over 0.2% but differ from other sub-clades within lineage A from 1.8% to 4.2%. Two distinct subclades are shown also within lineage B and differed by 4 to 6.2%. Clades containing lineage A and B do not differ by more than 8.3%.

Discussion

Surveillance of *S. stercoralis* in dogs, including molecular identification of positive cases, should be an important part of health management that can lead to a reduction in the risk of the

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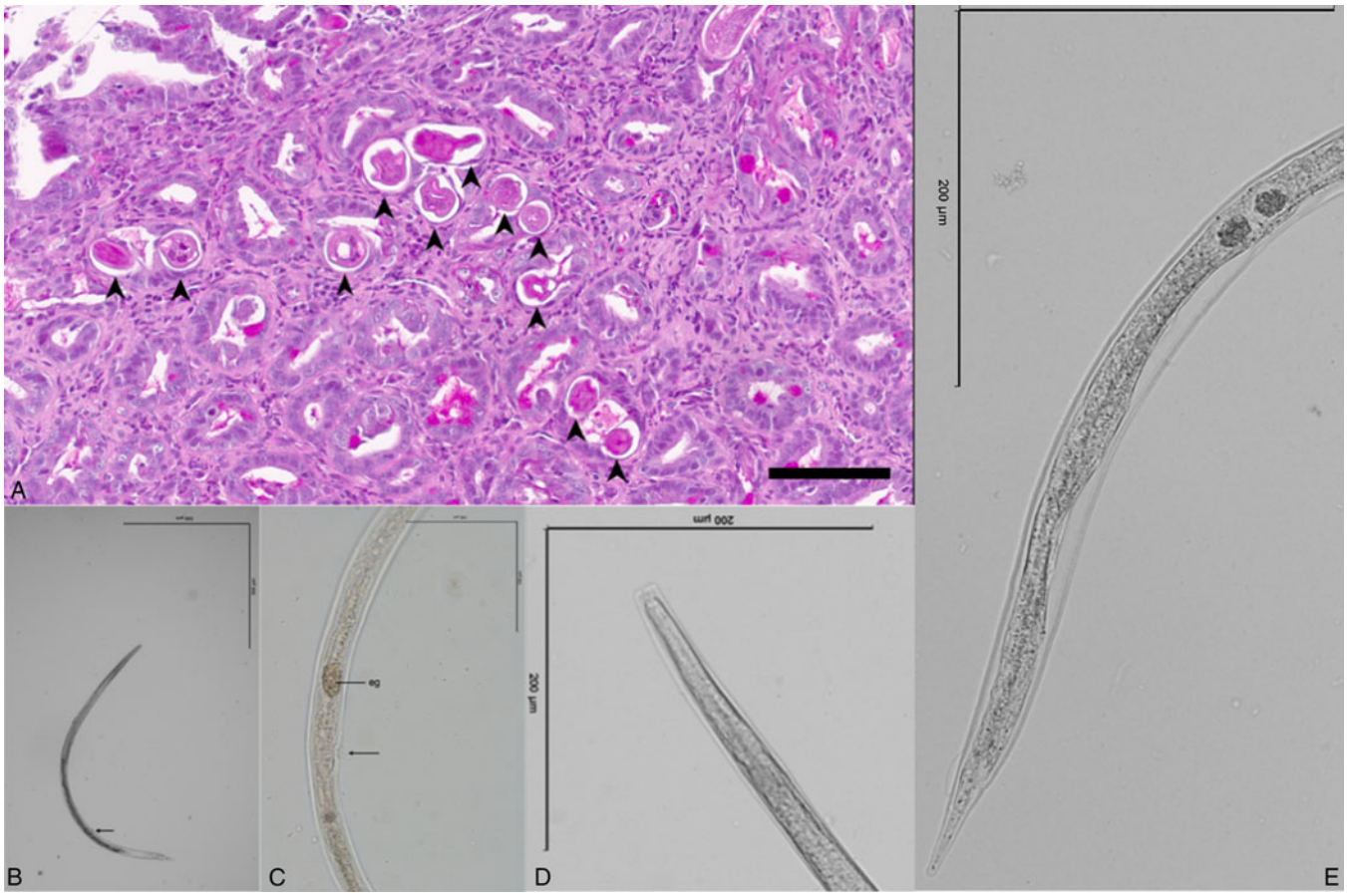


Figure 1. (A) Multiple cross sections of degenerated nematode parasites (arrows) embedded in the intestinal crypt, the remaining mucosal interstitial contains variable numbers of lymphocytes, plasma cells and scattered eosinophils, scale bar 80 µm; (B) general view of *Strongyloides stercoralis* parasitic female from dog, arrow indicates vulva, scale bar 500 µm; (C) vulval region of *S. stercoralis* parasitic female, arrow indicates vulva, scale bar 100 µm, abbreviation: eg – egg; (D) buccal cavity of *S. stercoralis* parasitic female, scale bar 200 µm and (E) tail of *S. stercoralis* parasitic female, scale bar 200 µm.

parasite's impact on host health, as well as a reduction in fatal cases. Knowledge of particular *S. stercoralis* haplotypes/lineages helps evaluate the risk of zoonotic transmission to humans (Nagayasu *et al.*, 2017). However, the combination of coproscopic and genetic analyses is not routinely performed in dogs. Here we present a case report of disseminated *S. stercoralis* infection in a dog evaluated by a combination of histology, microscopy and HTS approaches, with an appeal for the correct treatment approach.

Infections of dogs with *S. stercoralis* are considered rare in the Czech Republic. Only one case has been documented in the literature, reporting unidentified *Strongyloides* eggs found in dog faeces (Valkounová, 1982). Since only *S. stercoralis* L1 are typically detected in faeces of infected dogs, evaluation of this case is speculative. Recently, 9-week-old Chihuahuas imported to Switzerland from a Czech breeding station, were tested positive for *S. stercoralis*, suggesting that the infection had already been introduced from the Czech Republic (Schnyder *et al.*, 2022). Overall, cases of canine strongyloidiasis have emerged throughout Europe in the last decade (e.g. Dillard *et al.*, 2007; Riggio *et al.*, 2013; Eydal and Skírnisson, 2016; Paradies *et al.*, 2017; Bourgoin *et al.*, 2018; Basso *et al.*, 2019; Hall *et al.*, 2020; Schnyder *et al.*, 2022; Unterköfler *et al.*, 2022; Deak *et al.*, 2023) suggesting an increasing occurrence or recent spread across Europe, likely due to the importation of dogs (Schnyder *et al.*, 2022), including from *Strongyloides*-endemic countries (Unterköfler *et al.*, 2022). However, improved awareness, diagnosis and immunosuppression in dogs may also play a role in the increased number of cases (Cervone *et al.*, 2016). The dog in this study was adopted from Slovakia, which is considered one of the endemic countries for *Strongyloides* in both dogs and humans (Štrkolcová *et al.*, 2017). In addition, transmission of *S. stercoralis* between humans and dogs was suspected as IgG antibodies against *S. stercoralis* were detected in serum samples from children and rhabditiform larvae of *S. stercoralis* were detected in dogs in a Roma settlement in Slovakia (Štrkolcová *et al.*, 2017), although without molecular data. Further reports of strongyloidiasis in humans, but without molecular data, have been reported mainly from Eastern and Southern Europe (Ottino *et al.*, 2020), possibly influenced by increasing global migration (Asundi *et al.*, 2019; Marrone *et al.*, 2023).

Although most case reports and prevalence studies of infected dogs with *S. stercoralis* were based on microscopic diagnostics, some studies also included the genotyping of *Strongyloides* (Basso *et al.*, 2019; Salant *et al.*, 2021; Unterköfler *et al.*, 2022). Unfortunately, the haplotypes/lineages are not always reported, as in the case of Chihuahuas imported into Switzerland from the Czech Republic (Schnyder *et al.*, 2022). So far, only haplotype/lineage A has been detected in dogs in Europe, including the presented case (Nosková *et al.*, 2024). In the Czech Republic, the potentially zoonotic *S. stercoralis* haplotype A was also molecularly confirmed in captive orangutans kept in zoological gardens (Nosková *et al.*, 2023). In humans in Europe, haplotype A has only been confirmed in Italy (Barratt *et al.*, 2019).

Until now, studies on *Strongyloides* infection in dogs carried out in Europe have been conducted based on classical sequencing, which has its limitations when it comes to detecting multiple

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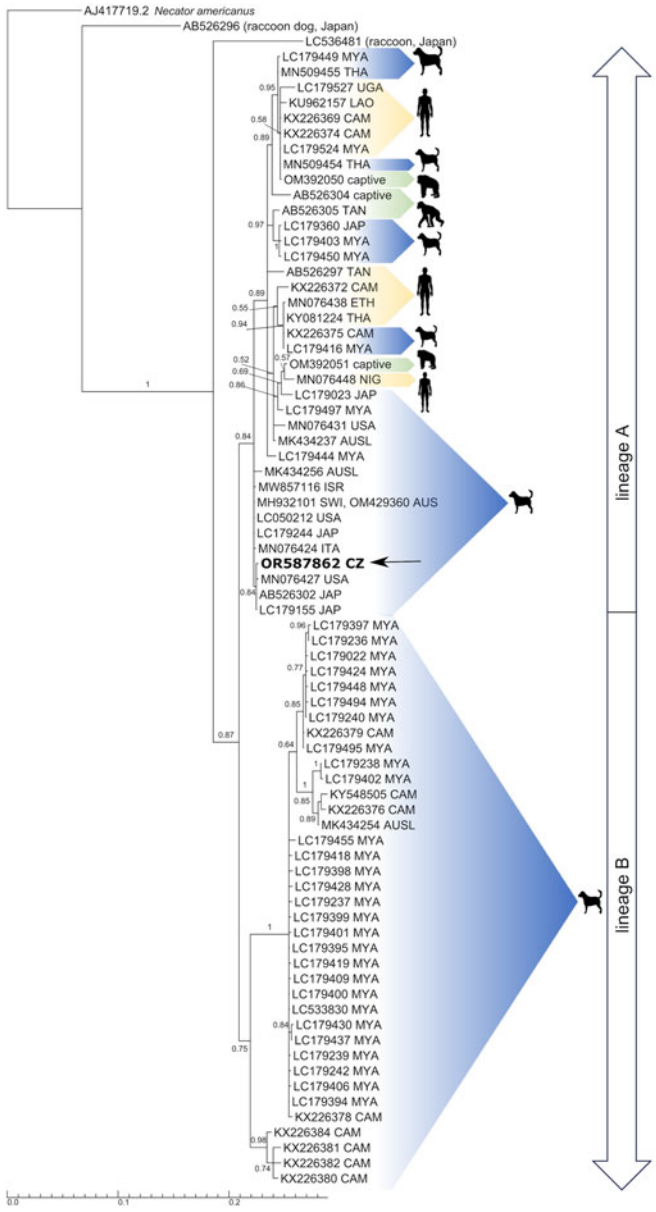


Figure 2. Bayesian inference phylogenetic tree inferred from cox1 (715 bp) calculated from a muscle-constructed alignment using GTR + G model for nucleotide substitutions. Numbers at the branches indicate Bayesian posterior probability based on 10^6 replicates. Branch lengths indicate expected numbers of substitutions per nucleotide site. Sequence obtained during the current study is in bold.

haplotypes. Conventional PCR analyses followed by Sanger sequencing provide only a single sequence and are therefore suitable for detection of *Strongyloides* from a single larva, for example, but the molecular identification of *Strongyloides* from total DNA extracted from faeces, which may contain multiple *Strongyloides* haplotypes, requires more complex genotyping approaches (Nosková *et al.*, 2024). Hypothetically, the almost complete absence of haplotype B could be due to the dominance of haplotype A in potentially mixed infections, which is preferentially detected by Sanger sequencing. Though, only the potentially zoonotic haplotype A was detected in our study when HTS was applied, and the previous fatal cases of disseminated strongyloidiasis analysed by molecular tools also revealed only the potentially zoonotic haplotype A so far. (Basso *et al.*, 2019; Unterköfler *et al.*, 2022). It appears that haplotype A is pathogenic or at least pathogenic under certain conditions, while the pathogenicity of haplotype B infecting dogs is so far unknown. However, in many studies, the haplotype was not identified, and it should be considered in the future. Perhaps if veterinary clinics, together with specialized institutions (e.g. universities, genomic or research centres), have the opportunity to perform HTS identification, the valuable results would fill the gaps regarding the genetic diversity and distribution of *Strongyloides* infection and clarify the possible pathogenicity of haplotype B.

In general, *S. stercoralis* disease progression in dogs varies from a latent course, especially in adult immunocompetent dogs, to fatal cases in young or immunocompromised dogs (Cervone *et al.*, 2016). Clinical signs include diarrhoea, erythema, respiratory problems, epaxial atrophy, nervous signs such as paresis, tremor, comatose states, recurrent epileptiform seizures, torticollis (Dillard *et al.*, 2007; Basso *et al.*, 2019; Schnyder *et al.*, 2022; Unterköfler *et al.*, 2022) or opisthotonus as in our study. Respiratory symptoms are attributed with *Strongyloides* infection as L3 enter the bloodstream and migrate through the trachea into the intestine (Page *et al.*, 2018). Disseminated strongyloidiasis develops when the host is exposed to long-term autoinfection. *Strongyloides* larvae have previously been found post-mortem not only in the intestinal mucosa and respiratory tract, but also in the CNS, spleen, kidneys and muscles (Thamsborg *et al.*, 2016; Paradies *et al.*, 2017). Strongyloidiasis in humans has a similar course. Most infections are mild, but under certain conditions (e.g. if the patient is immunosuppressed) dissemination may occur. While the mild course of the disease is characterised by gastrointestinal, pulmonary and dermatological symptoms, the infection can lead to severe systemic disease with fatal consequences. In a disseminated form, the larvae can occur in any organ and lead to systemic bacterial sepsis (Hagelskjaer, 1994; Qu *et al.*, 2016; Karanam *et al.*, 2021). In the case of the dog described in this study, the necropsy was not authorised by the dog's owner. However, we detected typical pathologies in the duodenum, ileum and colon by endoscopy. In addition, we observed parasitic females in the faecal sample, which is rare and suggests disseminated disease (Basso *et al.*, 2019).

The treatment of strongyloidiasis has not yet been clarified, as efficacy has only been surveyed in a small number of dogs. Ivermectin and fenbendazole appears to be the most effective anthelminthics, although *S. stercoralis* infection in dogs is not listed in indications of any canine anthelminthics containing these substances (Paradies *et al.*, 2017; Schnyder *et al.*, 2022). Nevertheless, a single administration is not considered sufficient, as the excretion of the larvae in the faeces persists (e.g. Paradies *et al.*, 2017; Schnyder *et al.*, 2022) and a repetition of the therapy at different intervals is usually necessary (Thamsborg *et al.*, 2016). Single dose of ivermectin is particularly insufficient for disseminated infection (Buonfrate *et al.*, 2022). Although the examined dog was treated against intestinal parasites, the effect on *Strongyloides* infection was insufficient, although the dog was classified as negative after the first treatment. Control of *S. stercoralis* infection can only be considered successful if the faecal examination repeatedly shows a negative larvoscopy result (Schnyder *et al.*, 2022). Unfortunately, in most cases of disseminated strongyloidiasis, prolonged therapy is not possible due to the deteriorating condition of the patient (Basso *et al.*, 2019). Patients with the disseminated form often suffer from secondary bacterial infections, respiratory failure, requiring appropriate intensive care (Cervone *et al.*, 2016). The development of a disseminated form can be promoted by the use of glucocorticoids (Mansfield *et al.*, 1996). Even short courses (6–17 days) of corticosteroids in immunocompetent patients without underlying immunosuppressive conditions have even been associated with death (Krolewiecki and Nutman, 2019). If a *Strongyloides* infection is detected in a dog taking corticosteroids, their use must be ceased immediately as there is a risk of development of the disseminated form

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(Graham *et al.*, 2019). The fatal case of the disseminated form in the examined dog was probably triggered by the prescription of corticosteroids. However, the disseminated infection in the studied dog developed before the administration of corticosteroids, when the dog showed opisthotomus, as the central nervous system can also be affected in a disseminated infection (Concha *et al.*, 2005).

Conclusion

The increasing occurrence of *S. stercoralis* in dogs in various European countries shows that this parasite appears to pose an increasing risk in Europe. The cross-border transportation of dogs from endemic countries and the accumulation of dogs in animal shelters could contribute to the spread of the infection. So far, only the potentially zoonotic haplotype/lineage A is known to be circulating in dogs in Europe, which in turn could pose a risk to human health. Due to the potential for cross-transmission between humans and dogs, *S. stercoralis* is a classic candidate for One Health approaches, where attention must be paid to accurate control and diagnosis in both humans and dogs. The correct treatment approaches should include: (1) Appropriate methods for the detection of *Strongyloides* infection, such as the Baermann larvoscopy or PCR, should be included in routine diagnostics in dogs. If faecal flotation is the only diagnostic method, the diagnostic reports should clearly state that the method performed cannot reliably detect *Strongyloides* so the veterinarians who commission the test are aware. (2) The repeated therapy and the confirmation of the treatment success should be stated only after several negative larvoscopy results. (3) If the *Strongyloides* infection is detected in a dog taking corticosteroids, their use must be ceased immediately as there is a risk of development of disseminated form. (4) Subsequently, appropriate molecular tools, ideally using HTS approaches, are highly recommended for *Strongyloides*-positive samples to raise awareness of the spread of individual *S. stercoralis* haplotypes/lineages. (5) General awareness of this spreading parasite should be raised among physicians, veterinarians and clinicians, together with caution in the use of glucocorticoids.

Data availability statement. Sequence based *cox1* of *Strongyloides stercoralis* is available and uploaded to GenBank under the accession numbers OR587862.

Acknowledgements. We acknowledge the CF Genomics CEITEC MU supported by the NCMG research infrastructure (LM2023067 funded by MEYS CR) for their support with obtaining scientific data presented in this paper. Computational resources were provided by the e-INFRA CZ project (ID:90254), supported by the Ministry of Education, Youth and Sports of the Czech Republic. The authors would like to thank Bethan Mason for language correction and improvement.

Author contributions. EN conceived the study, wrote the manuscript, performed the coproscopic examination, lab work, and phylogenetic analysis, VS wrote the article, performed the coproscopic examination, VH performed the veterinary examination, AC-E performed the histological examination, TK performed the lab work, VI performed statistical analyses, DM reviewed and edited the manuscript, BP supervised the project and wrote the manuscript.

Financial support. This project was supported from Masaryk University (MUNI/A/1602/2023).

Competing interests. The authors declare that no competing interests exist.

Ethical standards. Not applicable.

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Ilík, V., Schwarz, E.M., Nosková, E., Pafčo, B.

2024

Hookworm genomics: dusk or dawn?

Trends Parasitol. 40, 452–465. https://doi.org/10.1016/j.pt.2024.04.003

CellPress

Trends in Parasitology

Opinion

Hookworm genomics: dusk or dawn?

Vladislav Ilík [1,2,*], Erich M. Schwarz [3], Eva Nosková [1,2], and Barbora Pafčo [1,*]

Hookworms are parasites, closely related to the model nematode *Caenorhabditis elegans*, that are a major economic and health burden worldwide. Primarily three hookworm species (*Necator americanus*, *Ancylostoma duodenale*, and *Ancylostoma ceylanicum*) infect humans. Another 100 hookworm species from 19 genera infect primates, ruminants, and carnivores. Genetic data exist for only seven of these species. Genome sequences are available from only four of these species in two genera, leaving 96 others (particularly those parasitizing wildlife) without any genomic data. The most recent hookworm genomes were published 5 years ago, leaving the field in a dusk. However, assembling genomes from single hookworms may bring a new dawn. Here we summarize advances, challenges, and opportunities for studying these neglected but important parasitic nematodes.

Highlights

Hookworm infections are a major economic and health burden worldwide, but are also a neglected and often overlooked tropical disease.

Accurate hookworm diagnostics is difficult, and requires expertise in morphology and DNA-based approaches.

Hookworms are very diverse, but genetic and genomic data are sparse, incomplete, and few in number.

Assembly of four hookworm genomes was a breakthrough and has advanced nematode research.

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Pafčo, B., Kreisinger, J., Čížková, D., Pšenková-Profousová, I., Shutt-Phillips, K., Todd, A., Fuh, T., Petrželková, K.J., Modrý, D.

2019

Genetic diversity of primate strongylid nematodes: do sympatric nonhuman primates and humans share their strongylid worms?

Mol. Ecol. 28, 4786–4797. https://doi.org/10.1111/mec.15257

Check for updates

Received: 26 June 2019 | Revised: 22 September 2019 | Accepted: 24 September 2019

DOI: 10.1111/mec.15257

ORIGINAL ARTICLE

MOLECULAR ECOLOGY WILEY

Genetic diversity of primate strongylid nematodes: Do sympatric nonhuman primates and humans share their strongylid worms?

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Funding information
Czech Science Foundation, Grant/Award Number: 15-05180S; Institute of Vertebrate Biology, Czech Academy of Sciences, Grant/Award Number: RVO:68081766; CEITEC-Central European Institute of Technology, Grant/Award Number: CZ.1.05/1.100/02.0068; European Social Fund, Grant/Award Number: CZ.1.07/2.3.00/20.0300

Abstract

The close phylogenetic relationship between humans and nonhuman primates (NHPs) can result in a high potential for pathogen exchange. In recent decades, NHP and human interactions have become more frequent due to increasing habitat encroachment and ecotourism. Strongylid communities, which include members of several genera, are typically found in NHPs. Using optimized high-throughput sequencing for strain-level identification of primate strongylids, we studied the structure of strongylid communities in NHPs and humans co-habiting a tropical forest ecosystem in the Central African Republic. General taxonomic assignment of 85 ITS-2 haplotypes indicated that the studied primates harbour at least nine genera of strongylid nematodes, with *Oesophagostomum* and *Necator* being the most prevalent. We detected both host-specific and shared strongylid haplotypes. Skin-penetrating *Necator gorillae* haplotypes were shared between humans and gorillas but *Necator americanus* were much more restricted to humans. Strongylid communities of local hunter-gatherers employed as trackers were more similar to those of gorillas compared to their relatives, who spent more time in villages. This was due to lower abundance of human-origin *N. americanus* in both gorillas and trackers. Habituated gorillas or those under habituation did not show larger overlap of strongylids with humans compared to unhabituated. We concluded that the occurrence of the human-specific strongylids in gorillas does not increase with direct contact between gorillas and humans due to the habituation. Overall, our results indicate that the degree of habitat sharing between hosts, together with mode of parasite transmission, are important factors for parasite spillover among primates.

KEYWORDS
metabarcoding, primate, strongylid nematode, sympatric

Barbora Pafčo and Jakub Kreisinger contributed equally to this work.

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Ilík, V., Kreisinger, J., Modrý, D., Schwarz, E.M., Tagg, N., Mbohli, D., Nkombou, I.C., Petrželková, K.J., Pafčo, B.

2023

High diversity and sharing of strongylid nematodes in humans and great apes co-habiting an unprotected area in Cameroon.

PLoS Negl. Trop. Dis. 17, e0011499. https://doi.org/10.1371/journal.pntd.0011499

PLOS NEGLECTED TROPICAL DISEASES

RESEARCH ARTICLE

High diversity and sharing of strongylid nematodes in humans and great apes co-habiting an unprotected area in Cameroon

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OPEN ACCESS

Citation: Ilík V, Kreisinger J, Modrý D, Schwarz EM, Tagg N, Mbohli D, et al. (2023) High diversity and sharing of strongylid nematodes in humans and great apes co-habiting an unprotected area in Cameroon. PLoS Negl Trop Dis 17(8): e0011499. https://doi.org/10.1371/journal.pntd.0011499

Editor: Marcelo Larami Santoro, Instituto Butantan, BRAZIL

Received: September 21, 2022

Accepted: July 3, 2023

Published: August 25, 2023

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Data Availability Statement: Sequencing data are archived in the European Nucleotide Archive under the accession number of the whole project PRJEB64582. Accession numbers for each sample are available alongside related metadata in supporting information table S1 Table.

Funding: This work was supported by Czech Science Foundation (18-24345S awarded to D. M. and K. P. and 22-16475S awarded to K. P.), Fulbright Foundation (fellowship number PS00299111 awarded to V. I.) and Czech-American

Abstract

Rapid increases in human populations and environmental changes of past decades have led to changes in rates of contact and spatial overlap with wildlife. Together with other historical, social and environmental processes, this has significantly contributed to pathogen transmission in both directions, especially between humans and non-human primates, whose close phylogenetic relationship facilitates cross-infections. Using high-throughput amplicon sequencing, we studied strongylid communities in sympatric western lowland gorillas, central chimpanzees and humans co-occurring in an unprotected area in the northern periphery of the Dja Faunal Reserve, Cameroon. At the genus level, we classified 65 strongylid ITS-2 amplicon sequencing variants (ASVs) in humans and great apes. Great apes exhibited higher strongylid diversity than humans. *Necator* and *Oesophagostomum* were the most prevalent genera, and we commonly observed mixed infections of more than one strongylid species. Human strongylid communities were dominated by the human hookworm *N. americanus*, while great apes were mainly infected with *N. gorillae*, *O. stephanostomum* and trichostrongylids. We were also able to detect rare strongylid taxa (such as *Ancylostoma* and *Ternidens*). We detected eight ASVs shared between humans and great apes (four *N. americanus* variants, two *N. gorillae* variants, one *O. stephanostomum* type I and one *Trichostrongylus* sp. type II variant). Our results show that knowledge of strongylid communities in primates, including humans, is still limited. Sharing the same habitat, especially outside protected areas (where access to the forest is not restricted), can enable mutual parasite exchange and can even override host phylogeny or conserved patterns. Such studies are critical for assessing the threats posed to all hosts by increasing human-wildlife spatial overlap. In this study, the term "contact" refers to physical contact, while "spatial overlap" refers to environmental contact.

Scientific cooperation (LTAUSA18209) from the Ministry of Education, Youth and Sports of the Czech Republic awarded to K. P. Additional support was obtained from Masaryk University (MUNI/A/1488/2021 and MUNI/A/1422/2022) and Institute of Vertebrate Biology, Czech Academy of Sciences (RVO:68081766). The authors acknowledge Core Facility Genomics within CEITEC, Masaryk University supported by the NCMG research infrastructure (LM2018132) for their support with obtaining scientific data presented in this paper. Computation resources were provided by the e-Infrastruktura CZ project (ID:90140), supported by the Ministry of Education, Youth and Sports of the Czech Republic. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing interests: The authors have declared that no competing interests exist.

Author summary

Strongylid nematodes are common intestinal parasites, infecting a wide range of mammalian taxa, including humans and non-human primates (NHPs). They have evolved to live in vertebrate hosts for months or years and form complex communities within these hosts. Heavy strongylid infections can cause severe intestinal inflammation, intestinal lesions, respiratory difficulties, iron deficiency anemia, weight loss, childhood stunting, and adverse birth outcomes. As human population and human settlements have grown rapidly in recent decades, people often live in close proximity to wildlife, allowing for cross-transmission of soil-transmitted helminths. Because humans and NHPs (especially great apes) are closely related to one another evolutionarily, it is easy for strongylids infecting one primate species to jump to a different primate host. This evolutionary relationship can result in overlap and reciprocal exchange of pathogens and can have a damaging effect both on humans and NHP endangered species populations. As DNA-based diagnosis has become a routine part of modern parasitology, we employed a modern high-throughput sequencing approach to describe complex communities and zoonotic patterns of parasitic strongylid nematodes infecting humans and great apes in Cameroon.

Introduction

Among parasites, strongylid nematodes are of high importance to research, because they cause one of the most common but neglected tropical diseases in humans associated with the occurrence of pathologies [1,2]. They also cause significant parasitosis in livestock, which has a major economic impact on the livestock industry worldwide. Furthermore, under certain circumstances, strongylid nematodes could be pathogenic to wildlife, including non-human primates (NHPs) [3–5]. Strongylid nematodes inhabit various parts of the host body, mainly gastrointestinal and pulmonary tract, where they feed on blood or tissues [3,6,7]. They can live for many years within their hosts and generally do not cause mortality; however, severe infections can lead to inflammatory reactions, lesions, severe weight loss, anemia or malnutrition [8] and can be attributed to cases of human as well as animal deaths [9]. In humans, the most important strongylids are hookworms (*Necator americanus*, *Ancylostoma duodenale*, and *A*. *ceylanicum*), infecting over 400 million people worldwide [10]. *Necator* hookworms and the nodule worm of the genus *Oesophagostomum* are considered the most prevalent helminths in great apes [11].

Unfortunately, identification of distinct strongylid taxa from feces using microscopy is essentially impossible, as strongylid eggs are morphologically indistinguishable [5]. Thus, strongylid identification has been mostly dependent on DNA amplification and sequence analyses [12–14]. Strongylids have mainly been genotyped through DNA amplification methods targeting only one strongylid genus, followed by Sanger sequencing. However, occurrence of complex strongylid communities makes utilization of high-throughput sequencing (HTS) essential [4,15,16]. Despite some limitations (e.g., sequencing errors, short length of the reads or increased diversity due to presence of paralogues in genomes), HTS of standard phylogenetic markers amplified from complex target populations (metabarcoding) is inexpensive and allows efficient genotyping of hundreds of samples at a time, untangling mixed infections and detecting rare taxa [17–21]. Exact delineation of amplicon sequencing variants (ASVs) can help understand the molecular epidemiology of pathogens and, consequently, HTS metagenomics has brought about a much deeper insight into the diversity of strongylid nematodes and has revealed hidden zoonotic transmissions or parasite sharing [16,22–24].

The close phylogenetic relationship between NHPs and humans significantly facilitates the overlap and transmission of pathogens and can have a damaging effect on populations of both humans and endangered NHPs [25,26]. The rapid growth of the human population and the resulting encroachment into and modification of natural animal habitats have led to an increase in physical contact and spatial overlap with wildlife, creating ideal conditions for pathogen transmission and exchange also due to changes in ecological, political, economic, and social relations [27–29]. Recently, conservation activities and tourism also contribute to transmission of human pathogens to wildlife and can threaten endangered animals [30,31]. Therefore, it is critical to monitor pathogens, including parasites, at the human-wildlife interface to detect and find ways to prevent such exchanges. Several studies have revealed the zoonotic potential of strongylid nematodes with respect to various anthropogenic disturbances; for example, *Oesophagostomum* species were found to be shared between humans and great apes in Eastern Africa [12,32] and at least two *Necator* species are shared in Central African Republic and Gabon [33,34]. Using the HTS approach, Pafčo et al. [4] observed hidden transmissions of strongylid nematodes between humans and NHPs in the forest habitats of the Central African Republic, with *Necator* spp. as a main driving force of overlap between different hosts.

We explored strongylid nematode diversity in humans and great apes cohabiting an unprotected area in the northern periphery of the Dja Faunal Reserve, Cameroon. We evaluated possible zoonotic transmission patterns and assessed the impact of behavioral/hygiene habits of the local people on their strongylid infections. We employed an ITS-2 metabarcoding approach and predicted differences in strongylid nematode communities between different primate hosts.

Methods

Ethics statement

The research complied with the legal requirements of the Cameroon and was approved by Ministère de la Recherche Scientifique et de l'Innovation (permit number 0000105/MINRESI/B00/C00/C10/C12) and Ministère des Forêts et de la Faune (permit number 1371/PRS/MINFOF/SG/DFAP/SDVEF/SC). The ape samples were collected noninvasively and did not affect the animals. Human sampling and data collection followed the protocol approved by the Ethics Committee of the Biological Centre of Academy of Sciences, České Budějovice, Czech Republic and was approved by the local authorities. Sampling was performed after obtaining oral and written informed consent of all registered volunteers. Samples were numbered, paired with questionnaires and anonymized.

Study site, sample collection

Our study took place in the northern periphery of the Dja Faunal Reserve (Dja FR), located in South-East Cameroon. The reserve is part of a semi-deciduous lowland forest (500–700 m above sea level) with an equatorial and humid climate characterized by one short and one long dry season in between two rainy seasons (February–July/August–November) [35]. The unprotected area (40 km^2), comprising the target area of Project Grands Singes (PGS), under Antwerp Zoo Society, Belgium included the research camp La Belgique and three village settlements approximately 25 km from the camp. Several ethnic groups (including the Badjoué, the Fang, the Kaka, the Nzime, the Niem and the Baka) live in the periphery of the reserve in close coexistence with wildlife [36]. Although the human population density is low, the pressure on the reserve is substantial, as crops [37], hunting [38,39] and logging [40] remain the main sources of livelihood for the local people. High densities of central chimpanzees (*Pan*

troglodytes troglodytes) and western lowland gorillas (*Gorilla gorilla gorilla*) were recorded in the reserve as well as in the unprotected area around the camp La Belgique [41].

Human sampling was carried out in three villages–Duomo-Pierre, Malen V, and Mimpala (61 households and approximately 600 people in total)–and great ape samples were collected in secondary forest areas between the villages and around La Belgique research camp during September and October 2014 (major wet season peak). Fresh fecal samples (total number: n = 139) were collected non-invasively from humans (n = 48), median age 26 years, and free-ranging great apes: central chimpanzees (n = 31) and western lowland gorillas (n = 60). Human participants were provided with sampling tubes and samples were then gathered by researchers in the villages. Samples of great apes were collected from the ground under morning nests, a maximum of three hours after individuals left their nests. To reduce the risk of re-sampling of the same individuals and groups of individuals, only groups of different sizes (at the same locality) or groups of the same size (but not at the same locality) were sampled, and one sample per nest was taken. The samples were immediately fixed in 96% ethanol and stored at room temperature for a maximum of two weeks until they were sent to the Department of Pathology and Parasitology of University of Veterinary Sciences Brno, where they were stored at -20˚C.

Human participants also filled out a close ended questionnaire (S1 Fig) about their lifestyle including frequency of entering the forest, interaction with great apes, clothing, hygiene, anthelmintic treatment and dietary habits (Table 1). All participants spoke French and researchers assisted them to fill in the questionnaires.

DNA isolation, library preparation, sequencing

First, we took approximately 0.25 g of fecal sample preserved in ethanol and evaporated the ethanol overnight at 37˚C. We extracted total genomic DNA from dry fecal samples using PowerSoil DNA isolation kit (MO BIO Laboratories, Qiagen company, USA) and amplified ribosomal DNA (rDNA), specifically the variable section of rDNA (internal transcribed spacer 2; ITS-2). We prepared sequencing libraries according to the protocol of Pafčo et al. [16], using two-step PCR following the Fluidigm Access Array primer design. We processed each sample in duplicate and included two negative and three positive controls according to the protocol. We sequenced the final libraries using the Illumina MiSeq platform (Illumina MiSeq Reagent Kit v2, sequencing 500 cycles of 2 x 250 bp paired-end reads). Additionally, we created a large metadata table containing sample identification (ID), collection site and host species.

Table 1. Results of questionnaires based on respondents' answers. Survey focused mainly on human-animal interactions, lifestyle and hygiene standards.

Activity	Yes	No	Frequently	Sometimes	Never	River	Well	Both
Entering the forest	-	-	64.6%	29.2%	6.2%	-	-	-
Contact/Encounter with wild apes	89.6%	10.4%	-	-	-	-	-	-
Contact with ape feces	70.8%	29.2%	-	-	-	-	-	-
Wild apes around household	22.9%	77.1%	-	-	-	-	-	-
Wearing shoes in the forest	39.6%	60.4%	-	-	-	-	-	-
Eating from the ground	97.9%	2.1%	-	-	-	-	-	-
Washing crops before eating	8.3%	91.7%	-	-	-	-	-	-
Washing hands before eating	37.5%	62.5%	-	-	-	-	-	-
Drinking from water sources	-	-	-	-	-	89.6%	2.1%	8.3%
Anthelmintic treatment	29.2%	70.8%	-	-	-	-	-	-
Taking plant-based drugs	4.2%	91.7%	-	-	-	-	-	4.1%

https://doi.org/10.1371/journal.pntd.0011499.t001

Data processing and statistics

We trimmed raw.fastq sequences using Skewer [42] and followed by paired-end reads assembly in PEAR merger [43]. We eliminated low quality sequences (with expected error rate > 1%) from the dataset. We detected ITS-2 amplicon sequencing variants (ASVs) and estimated sample relative abundances using software dada2 [44]. Using dada2's algorithm, sequences inconsistently present in both duplicates were marked as potential artifacts (e.g., sequences with low template content, chimeras or sequencing errors) and removed from downstream analyses (5–7% of sequences after quality control). We searched for corresponding sequences via standalone BlastN (performed on the NCBI nt database, which was downloaded on 10th February 2020); we excluded environmental or uncultured samples from the database and filtered out all blast hits with < 85% identity and < 90% coverage from the file. We downloaded taxonomy for blast hits using taxize package [45], and used the created reference database to assign a taxonomic classification in our dataset via dada2's AssignTaxonomy method, implementing a Naïve Bayesian Classifier algorithm [46].

We merged the resulting taxonomy table with our metadata table in RStudio (https://www.rstudio.com); into a single phyloseq object, suitable for downstream analyses. We executed all data analyses in the statistical software RStudio. We de-noised the raw dataset (variants unclassified up to "family" level and "non-strongylid" were removed from the dataset) and used a generalized linear model (GLM) with quasipoisson error distribution to test differences in alpha diversity, evaluated as number of ASVs per sample, among the studied hosts. Additionally, we employed post-hoc testing (Tukey) to identify levels of factorial response that differ from each other. Moreover, we measured the alpha diversity by Shannon's and Simpson's indexes; we defined community composition as prevalence and relative representation of ITS-2 ASVs using Jaccard and Bray-Curtis ecological distances. In order to prevent negative eigenvalues during computation, we performed square root transformation of the dataset. We then performed Principal coordinate analysis (PCoA) on both Jaccard and Bray-Curtis dissimilarities. To test the interspecific differences in strongylid nematode community compositions among the hosts, we executed permutational analysis of variance (PERMANOVA), followed by analysis of similarity (ANOSIM). We implemented Multivariate general linear models (GLMs) from the R package mvabund [47] to search for community-wide divergence and identification of significant ASVs that varied due to the different host species effect. For better resolution, we constructed a diagram showing proportion of reads for significant variants. We further implemented GLM testing with quasipoisson error distribution, followed by PERMANOVA and ANOSIM to evaluate the impact of all factors from the questionnaires (Table 1) on the strongylid alpha and beta diversity in humans.

Results

Overall characteristics of the dataset

We analyzed fecal samples of humans (n = 48), western lowland gorillas (n = 60) and central chimpanzees (n = 31). In total, 2,943,087 high-quality reads were identified, with a median sequencing depth per sample of 15,612 (minimum = 9, maximum = 375,905). After duplication of the obtained sequencing data from negative control samples, no ITS-2 strongylid reads were found. Taxonomic assignment revealed 65 ITS-2 amplicon sequencing variants (ASVs), including at least five strongylid genera (Table 2). Thirty-two unassigned variants (present in 45% of samples) were tentatively classified as being closest to *Nematodirus* sp. or *Travassostrongylus* sp.; however, the sequence identity and match scores were low (84.1% and 76.8%,

Table 2. List of identified strongylid nematodes found in studied hosts, sequences NCBI Accession numbers and reference.

Family	Genus	Species	NCBI Accession	Reference
Chabertiidae	*Oesophagostomum*	*Oesophagostomum stephanostomum* type I	KR149648.1	Cibot et al. [12]
	Oesophagostomum	*Oesophagostomum stephanostomum* type II	AB821022.1	Makouloutou et al. [62]
	Oesophagostomum	*Oesophagostomum* sp.	KR149658.1	Cibot et al. [12]
	Ternidens	*Ternidens deminutus*	AJ888729.1	Schindler et al. [64]
Ancylostomatidae	*Necator*	*Necator americanus*	LC088287.1, LC036563.1 MG256601.1	Hasegawa et al. [34]; Hasegawa *unpubl.*; Jariyapong & Punsawad *unpubl.*
	Necator	*Necator gorillae*	LC088299.1	Hasegawa et al. [34]
	Necator	*Necator* sp.	AB793535.1	Hasegawa et al. [33]
	Ancylostoma	*Ancylostoma* sp.[†]	LC036567.1	Hasegawa *unpubl.*
Trichostrongylidae	*Trichostrongylus*	*Trichostrongylus* sp. type I	Unassigned[‡]	NA
	Trichostrongylus	*Trichostrongylus* sp. type II	LC185220.1	McLennan et al.[66]
Unclassified	Unclassified	Unclassified	Unassigned[§]	NA

[†]Closest hit *A. ceylanicum* (similarity 95.5%)

[‡]Closest hits *T. vitrinius* (similarity 98.48%), and *T. colubriformis* (similarity 97.34%)

[§]Probably two taxa: closest hits *Nematodirus* sp. (similarity 84.1%) and *Travassostrongylus* sp. (similarity 76.8%)

https://doi.org/10.1371/journal.pntd.0011499.t002

respectively), thus those variants probably do not represent these genera and could possibly indicate novel nematode species in the sample.

Composition of strongylid communities

The most prevalent variants belonged to three genera: *Necator*, *Oesophagostomum* and *Trichostrongylus* (Table 3). A bar graph visualizing relative abundances of strongylid variants for

Table 3. List of numbers of identified amplicone sequence variants (ASVs), their proportion of total reads, numbers of infected hosts and ASV prevalence among host species.

Parasite taxa	Number of identified ASVs	Total reads proportion (%)	Number of ASVs in humans	Number of ASVs in gorillas	Number of ASVs in chimpanzees	Prevalence in humans (%)	Prevalence in gorillas (%)	Prevalence in chimpanzees (%)
Oesophagostomum stephanostomum type I	16	41.1	1	12	11	27.1	81.7	96.8
Oesophagostomum stephanostomum type II	3	0.7	0	2	3	0	6.5	35.5
Oesophagostomum sp.	1	> 0.1	0	1	0	0	1.7	0
Necator americanus	16	21.7	15	5	0	66.7	31.7	0
Necator gorillae	14	20.0	2	14	4	16.7	96.7	87.1
Necator sp.	8	0.1	0	6	3	0	13.3	9.7
Trichostrongylus sp. type I	3	0.2	0	3	0	0	13.3	0
Trichostrongylus sp. type II	2	6.3	1	2	1	2.1	76.7	61.3
Ancylostoma sp.	1	> 0.1	0	0	1	0	0	3.2
Ternidens deminutus	1	> 0.1	0	1	0	0	1.7	0
Unclassified	32	10.0	4	18	17	8.3	58.3	77.4

https://doi.org/10.1371/journal.pntd.0011499.t003

all studied individuals revealed interspecific differences in the composition of strongylid nematode communities according to host species (Fig 1). Humans were predominantly infected by *N. americanus* (66.7%; median relative abundance of reads x = 3,340, min. = 143, max. = 375,811), while *N. gorillae* variants were less common (16.7%; x = 361, min. = 116, max. =

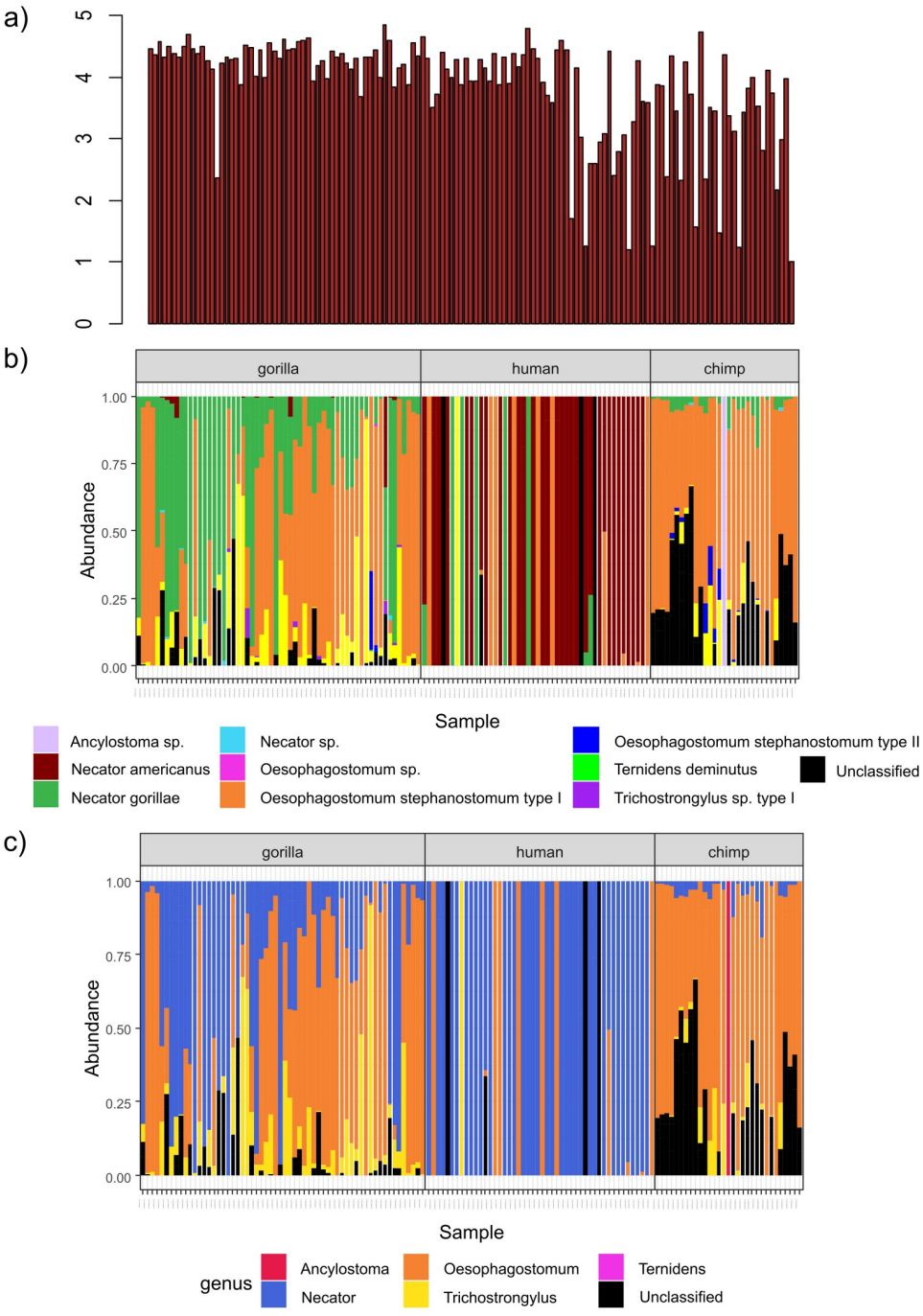


Fig 1. Bar plots showing a) number of reads in each individual sample on a log10 scale b) relative community composition of strongylid nematodes in examined samples at the species level, c) relative community composition of strongylid nematodes in examined samples at the genus level. Each column represents a sample. Numbers of reads (a) / relative abundances (b, c) of reads are depicted as color panels.

https://doi.org/10.1371/journal.pntd.0011499.g001

18,068). A significant portion of human infections also included *O. stephanostomum* type I (27.1%; x = 35, min. = 9, max. = 3,809). *Trichostrongylus* sp. type II (2.1%; 870 reads) and four unassigned variants (8.3%; x = 22, min. = 16, max. = 207) were also found in humans. Strongylids in great apes were dominated by variants of *N. gorillae* (overall prevalence 91.9%; x = 1,872, min. = 47, max. = 110,743), *Oesophagostomum stephanostomum* type I (89.3%; x = 13,209, min. = 15, max. = 49,181), *Trichostrongylus* sp. type II (69.0%; x = 863, min. = 16, max. = 38,774) and unassigned variants (67.9%; x = 3420, min. = 63, max. = 32,817). *Necator americanus* variants were found only in gorillas (31.7%; x = 107, min. = 29, max. = 7,646), while there was no evidence for *N. americanus* in chimpanzees. Additionally, unidentified variants of *Necator* species (neither *N. americanus* nor *N. gorillae*) were detected in great apes (13.3% in gorillas; 9.7% in chimpanzees; x = 130, min. = 50, max. = 252). Three taxa were recorded in low prevalence and with reads only evident in gorillas (*Oesophagostomum* sp. 400 reads; *Trichostrongylus* type I x = 688, min. = 52, max. = 1,832; and *Ternidens deminutus* 45 reads) and one taxon was detected only in a chimpanzee (*Ancylostoma* sp. 228 reads). We found eight ASVs shared between humans and great apes (8.25% of all observed ASVs), suggesting zoonotic transmission: two *N. gorillae* variants, one *O. stephanostomum* type I variant, and one *Trichostrongylus* sp. type II variant were found in humans, gorilla and chimpanzees, while four *N. americanus* variants were shared only between humans and gorillas.

Alpha and beta diversity

Variant diversity ($x' = 7$; min. = 1, max. = 17) differed among the studied hosts (GLM: $F_{(2,138)} = 203.36$, $p < 0.0001$). Variant diversity in humans was lower compared to both species of great apes (Tukey post-hoc testing: p = 0.0001 for all pairwise comparisons) (Fig 2), while there was no evidence of significant differences between gorillas and chimpanzees ($p > 0.3$). PCoA diagrams based both on Jaccard and Bray-Curtis ecological distances confirmed clear differences between humans and great apes in both composition and relative abundance of

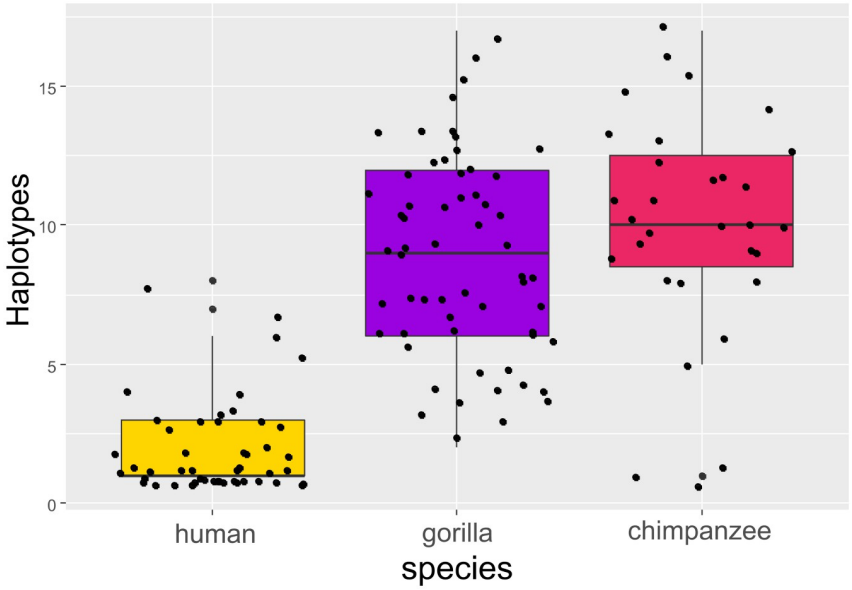


Fig 2. Alpha diversity of strongylid nematode communities, boxplot of amplicone sequencing variants (ASVs) counts for each sample (dots) according to host species. Different letters above boxes indicate statistically significant differences according to GLM test.

https://doi.org/10.1371/journal.pntd.0011499.g002

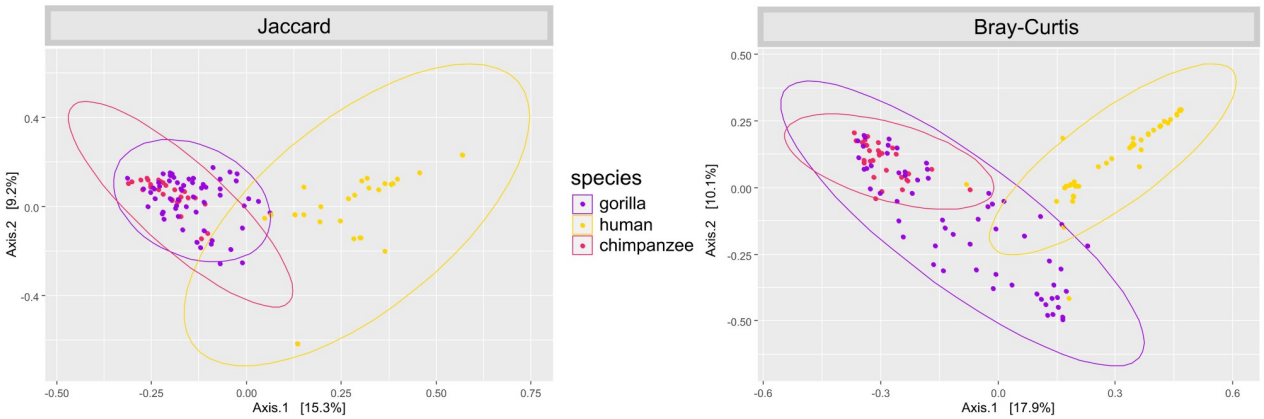


Fig 3. PCoA ordination diagrams of beta diversity of strongylid nematode communities based on Jaccard ecological distance: Presence/absence of amplicone sequencing variants (ASVs); Bray-Curtis ecological distance (relative abundances of reads).

https://doi.org/10.1371/journal.pntd.0011499.g003

strongylid ASVs (Fig 3). Significant differences between different host species in the composition of their strongylid nematode communities were further confirmed by PERMANOVA (Jaccard: $F_{(2,138)} = 11.655$, $p = 0.001$; Bray-Curtis: $F_{(2,138)} = 14.644$, $p = 0.001$) and ANOSIM (Jaccard: $R = 0.4456$, $p = 0.001$; Bray-Curtis: $R = 0.4204$, $p = 0.001$) tests. Tukey post-hoc testing revealed significant differences between humans and other great apes for both Jaccard and Bray-Curtis ($p < 0.01$ for all pair-wise combinations) distances. Within great apes, there was no statistically significant result for Jaccard ($p = 0.36$) indicating roughly the same composition of strongylid ASVs; however, results for Bray-Curtis indicated differences in relative abundances (proportion) of ASVs between great apes ($p < 0.001$). Mvabund testing confirmed the interspecific differences (mvabund: $\Delta DF = 2$, $\chi 2 = 1002.371$, $p = 0.001$) and identified 17 ITS-2 ASVs with whose different relative abundances were the main driving force of diversity between different host species in contrast to shared haplotypes (Fig 4a and 4b). Differences among hosts were mainly due to greater frequencies of *O. stephanostomum*, *N. gorillae*,

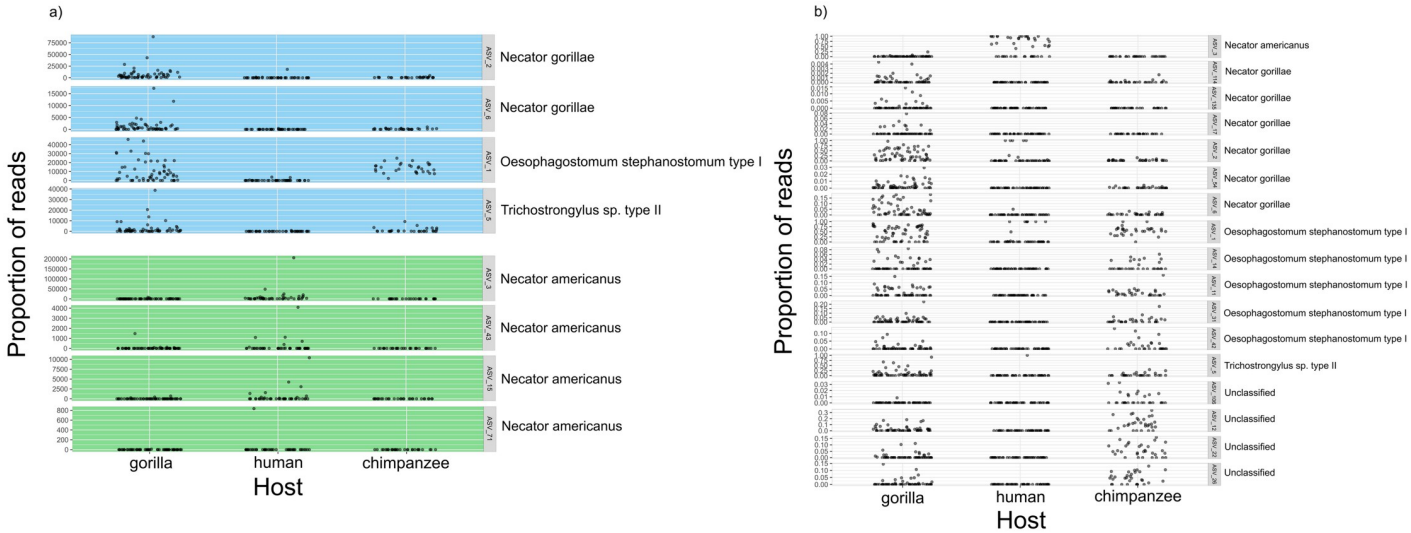


Fig 4. Plots showing relative abundance of ITS-2 amplicone sequencing variants (ASVs) indicated by a) shared ASVs between humans and great apes; ASVs shared between all three studied groups are highlighted in blue; ASVs shared between humans and gorillas are highlighted in green, b) Mvabund analyses as a driving force of differences among studied hosts.

https://doi.org/10.1371/journal.pntd.0011499.g004

Trichostrongylus type II and unclassified strongylids in great apes, whereas *N. americanus* was most frequent in humans.

We found no significant impact of behavioral or hygiene habits of the local people on either strongylid alpha or strongylid beta diversity (GLM: $p > 0.05$; PERMANOVA: $p > 0.05$; ANOSIM: $p > 0.05$).

Discussion

Strongylid community composition

We explored strongylid diversity and transmission patterns in humans and great apes sharing the same habitat in an unprotected area at the northern border of the Dja Faunal Reserve (Dja FR), Cameroon. Using the ITS-2 locus for identification, general taxonomic assignment revealed 95 strongylid ITS-2 amplicon sequence variants (ASVs), of which we could classify 65 at the genus/species level. We are aware of the limitations of the ITS-2 marker [17–19] and attempted to eliminate errors by running all samples in duplicates, excluding inconsistently present sequences from the dataset and using negative controls. We did not set a threshold for the number of reads per sample to detect rare strongylid taxa. We know that the threshold in this case is somewhat controversial (due to the low number of reads in some samples/ASVs). However, no official threshold has been set yet and different thresholds and settings are used in other studies [22,48].

In contrast to our previous study in Dzanga Sangha Protected Areas (DSPA), Central African Republic, where only two variants (from the total of 85) remained unassigned [4], our data from Dja FR contained 32 unassigned ASVs on the genus level. This suggests a more diverse strongylid fauna in Dja apes and humans, and further indicates that strongylid nematodes are a rather understudied group with unexplored diversity.

Overall, the composition of strongylid communities found in Dja remained generally consistent with previous studies, suggesting that *Necator* and *Oesophagostomum* are the most prevalent strongylid genera in African apes and humans [4,12,16,24,32,34,49,50], but unlike in previous studies, these were followed by *Trichostrongylus* and unassigned genera. Dja apes were mostly infected by variants of *O. stephanostomum* and *N. gorillae*, both commonly found in great apes [4,12,24,32,50]. Humans were mostly infected by *N. americanus* variants, confirming that *N. americanus* is the dominant human-specific hookworm in general [51]. Great apes exhibited higher strongylid diversity than humans and mixed infections of more than one strongylid species were frequently observed, which is consistent with previous findings in DSPA [4]. On the contrary, Vlčková et al. [52] used the same sample set as the present study and observed a higher alpha diversity of *Entamoeba* (protozoan parasite) communities in people living in the Dja compared to co-occurring great apes.

Mason et al. [24] employed HTS techniques to survey strongylid nematodes of wild western lowland gorillas in five distinct localities across the Congo Basin. The authors [24] observed lower strongylid diversity in western lowland gorillas in Dja compared to other study areas (including DSPA, CAR), and explained this to be due to the impact of greater anthropogenic disturbance on strongylid communities in the unprotected periphery of Dja FR compared to protected sites. Traditionally, parasites are thought to have negative effects on the host; however, they are a natural part of the host environment due to millions of years of evolution [53] and it has more recently been speculated that the loss of parasitic symbionts in industrialized human populations may contribute to an increase in autoimmune diseases [54]. It appears that a loss of parasitic symbionts occurs in areas of increased anthropogenic pressure [55]; however, studies employing better characterization of the anthropogenic disturbance across sites are warranted.

Necator

We discovered six ASVs of *Necator* spp. (6.19% from the total of 97 ASVs found) being shared between multiple hosts. Besides the human hookworm *N. americanus*, other *Necator* species (*N. exilidens*, *N. congolensis* and *N. gorillae*) have been reported in great apes [56–58] and also in humans in Africa [4,33,59]. Four variants of *N. americanus* and two variants of *N. gorillae* were found co-infecting humans and great apes, suggesting ongoing transmission events previously described in the tropical forest ecosystem in DSPA and Moukalaba-Doudou National Park [4,16,33,34]. While *N. americanus* variants were found mostly in humans, four of them were shared with gorillas; this demonstrates that *N. americanus* is not a solely human-specific parasite. Moreover, such a finding was previously observed in African great apes [4,24,33,34]. *Necator gorillae* variants were found predominantly in great apes, suggesting its probable ape origin, but they were also shared with humans. The *N. gorillae* variants corresponded to those previously found in gorillas in Gabon [34]. We did not find evidence of *N. americanus* infecting wild chimpanzees, which supports a previous hypothesis of a lower susceptibility of chimpanzees to *N. americanus* infections [34], despite some cases of chimpanzee infections having been previously recorded [4,60]. Additionally, we found a few variants of undetermined *Necator* sp. in Dja apes corresponding to variant III-1 first found in humans in DSPA by Hasegawa et al. [33], later reported in western lowland gorillas across several African localities [24,34]. Hasegawa et al. [33] speculated that variant III-1 sequences may represent *N. congolensis* or *N. exilidens*, previously described in chimpanzees [56,57]; however, the original descriptions of *N. congolensis* or *N. exilidens* were made at the beginning of the last century, and even "traditional" morphology-based taxonomy of *Necator* non-*americanus* species remain unclear [59]. Several *Necator* species are clearly capable of infecting both humans and NHPs, at least in habitats where they share the same environment. However, the exact species diversity is not known, nor is the epidemiology and ability (particularly of the non-*americanus* species) to spread in human populations. Therefore, large-scale studies covering multiple populations of wild great apes, other NHPs and humans, with utilization of advanced HTS tools combined with modern morphological characterizations will be required for better understanding of *Necator* epidemiology.

Oesophagostomum

Two *Oesophagostomum* species are commonly found in great apes and humans throughout Africa–*O. stephanostomum* [12,50] in great apes and *O. bifurcum* in humans, especially in West Africa [61]; other *Oesophagostomum* species have been recorded, but they are much rarer [11,13]. We recorded one variant of *O. stephanostomum* type I shared among great apes and humans in Dja, corresponding to the variant infecting NHPs and humans in Kibale, Uganda [12]. This means our finding is the second observation of *O. stephanostomum* in humans, providing evidence that *Oesophagostomum* species have zoonotic potential under suitable circumstances. Pafčo et al. [4] also reported *O. stephanostomum* infecting NHPs in DSPA but not in humans, thus suggesting its ape origin. This is also supported by Mason et al. [24], who found *O. stephanostomum* in high prevalence in western lowland gorillas across several African localities. We only found the second *Oesophagostomum* group (*Oesophagostomum stephanostomum* type II) in great apes; this group was previously described in western lowland gorillas in Moukalaba-Doudau National Park (MDNP), Gabon [62] and in Dja FR, Cameroon (using the same gorilla dataset as was used for this study [24]). We only found the other variants of undetermined *Oesophagostomum* sp. in one gorilla. They correspond to *Oesophagostomum* sequences from humans and NHPs in Kibale, Uganda [32], further recorded by Cibot et al. [12] in olive baboons in other part of Kibale, Uganda. Pafčo et al. [4], found *O. bifurcum*

infecting mangabeys in DSPA, but not great apes; we also found no evidence of this species in great apes or humans in Dja FR, although it is known to commonly infect both humans and NHPs in Africa [24,61,63].

Other strongylids

Other strongylid nematodes also infect humans and NHPs in Africa, such as the "false hookworm" *Ternidens deminutus*, the cyathostomine worm *Murshidia* spp. [64], strongylids belonging to Trichostrongylidae [4,65], and other pulmonary strongylids such as *Mammomonogamus* [7]. We found several ASVs of *Trichostrongylus* spp. being harbored by Dja FR great apes (their strongest BlastN matches were to trichostrongylids parasitic in sheep), and we found one variant to be shared between great apes and one human at Dja FR, corresponding to the *Trichostrongylus* variant from chimpanzees living in degraded forest fragments in Bulindi, Uganda [66]. Variants of *Trichostrongylus* were reported by Pafčo et al. [4] and Mason et al. [24] in lowland gorillas, and adult *Trichostrongylus* worms were found in necropsied mountain gorillas in Rwanda [67]. Although several cases of *Trichostrongylus* infections have been reported in humans in north-eastern Thailand, Lao People's Democratic Republic (PDR) and urban areas of Salvador City, Brazil [68,69], human *Trichostrongylus* infections are considered rather incidental. We found one variant of *Ternidens deminutus* infecting western lowland gorillas, closely similar to the one from Mona monkeys (*Cercopithecus mona*) found in Ghana [64]; this finding also corresponded to Pafčo et al. [4], who found four *T. deminutus* variants infecting great apes, being closely related to the same sequence. *T. deminutus* is considered to be a neglected parasite of humans [64] and has also been reported in chimpanzees of Tai, Côte d' Ivoire [5] and in western lowland gorillas of Loango National Park, Gabon and in DSPA, CAR by Mason et al. [24], thus raising questions about its origin and zoonotic potential. *Ancylostoma* duodenale is considered a human-specific parasite and was found by Pafčo et al. [4] in humans in DSPA, Central African Republic. Our data show evidence for the first chimpanzee infection by *Ancylostoma* sp. ever recorded; however, we could not specifically assign the variant to a known *Ancylostoma* species, and it was found in only one chimpanzee sample, representing 100% of total sample reads. Such homogeneity in chimpanzees is rather unusual, according to our dataset.

Zoonotic transmission patterns

In Dja FR, humans exhibited lower strongylid alpha diversity than great apes and formed a separate cluster distinct from great apes, which was caused by dominance of *N. americanus* variants in both prevalence and relative abundance (measured as the proportion of sequencing reads assigned to this species). On the other hand, the strongylid communities of the two great ape species overlapped and were dominated by variants belonging to *N. gorillae*, *O. stephanostomum*, *Trichostrongylus* type II and unclassified variants. Our results corroborate those from DSPA, CAR [4], where the composition of strongylid communities was also shaped by the extent of habitat sharing, which is much more intense among species of great apes than between humans and great apes. Infective larvae (L3) of monoxenous strongylid nematodes develop in the external environment [6], thus habitat sharing increases the risk of infection and transmission between hosts. Thus, in both DSPA and Dja FR, the observed patterns of strongylid communities did not reflect the phylogenetic relationships of the hosts as they are more similar between great ape species than between phylogenetically closer humans and chimpanzees [70]. Interestingly, the composition of human and chimpanzee *Entamoeba* communities in Dja FR overlapped, while that of gorillas formed a clearly separated cluster, displaying a pattern that reflects the phylogenetic distance between the hosts [52]. Mann et al.

[71] analyzed gut protists and nematodes of NHPs from various sites using the 18S phylogenetic marker. Although 18S markers cannot provide high phylogenetic resolution for strongylid nematodes [17], these results showed that gut eukaryotes (unlike symbiotic gut bacteria) were only weakly structured by primate phylogeny, similar to the case for gut mycobiome [72]. More studies are needed to understand the drivers shaping various eukaryotic gut communities of great apes and humans.

In Dja FR, we found no impact of frequency of entering the forest, interaction with great apes, clothing, hygiene, anthelmintic treatment or dietary habits; however, the implementation of questionnaires was a pilot activity, and a more detailed and rigorous social science approach would be needed to explain differences in strongylid infections among Dja FR humans. We recorded higher numbers of strongylid ASVs shared between humans and great apes in Dja FR in comparison to DSPA. This is quite surprising as the majority of human respondents in Dja FR were agriculturists while in DSPA the studied humans were contemporary BaAka hunter-gatherers and some were even employed as gorilla trackers for the Primate Habituation Programme [4,73]. Our results may therefore indicate a possible impact of rural people's lifestyles causing anthropogenic disturbance and subsequent changes in spatial overlap between apes and humans on strongylid transmission patterns. The northern periphery of the Dja FR experiences high anthropogenic pressure as the forest is degraded and fragmented, with intense logging, hunting and farming occurring in the area [37–40]. Conversely, in DSPA, the studied apes inhabited strictly protected parts, namely the Dzanga sector within Dzanga-Ndoki National Park in DSPA, CAR [4,16,24,33,74]. The agricultural fields of the northern periphery of Dja FR attract wildlife, including apes, which can result in crop-raiding, and both humans and apes can defecate around fields [37,75]. Local people often walk barefoot through Dja agricultural fields and eat crops straight from the ground without washing them (Table 1). Together with almost no anthelmintic treatment and poor sanitation and hygiene rules, the transmission of strongylid parasites can be greatly facilitated as *Necator*, *Oesophagostomum* and *Trichostrongylus* are parasites transmitted by skin penetration or oral ingestion [6]. People living in Sub-Saharan Africa have always shared their habitat with NHPs. Our results pertain to people with agricultural and hunter-gatherer lifestyles and apes inhabiting unprotected and protected areas, and indicate that ecological, social and even political economic changes resulting in greater pressures on wildlife habitats and changes in spatial proximity between wildlife and humans have created opportunities for intensified soil-transmitted helminth spillover in both directions.

Future research should include analyses of the strongylid communities of apes from multiple areas of varying conservation status. For example, it is necessary to sample gorillas from the special reserve in DSPA, multiple-use zone with human activities surrounding the national park and Bantu people following agricultural lifestyle co-habiting DSPA, to better understand the drivers of transmission patterns in various host cohorts [76,77]. Importantly, a multi-disciplinary and anthropological–historical approach, including social science parameters, should be implemented to describe the patterns of contact and spatial overlap of humans, apes and helminths across various localities [29].

Conclusion

We reveal complex strongylid nematode communities of great apes and humans sharing an unprotected tropical forest habitat in Cameroon. The great apes exhibited a greater diversity of the strongylid fauna harbouring more amplicon sequencing variants (ASVs) and rare variants in comparison to humans. *Oesophagostomum* and *Necator* were the dominant components of strongylid communities in all studied hosts, and the driving force of strongylid overlaps.

Human communities were dominated by *Necator americanus*; although generally thought to be human-specific, this parasite was also shared by gorillas. *Necator gorillae*, originally thought to be a parasite confined to NHPs, was widespread across all studied host species, including humans. We observed a second case of *O. stephanostomum* infection in humans. In contrast to previous studies conducted in the DSPA, CAR, we recorded more genera and variants being shared between humans and great apes, which might be due to significant anthropogenic pressure in the periphery of the reserve, which is not protected. Most African apes occur outside protected areas [78] and thus improving the effectiveness of pathogen monitoring, conservation efforts and management not only inside, but also outside, protected areas is urgently warranted.

Supporting information

S1 Fig. Questionnaire filled by human participants about their lifestyle including frequency of entering the forest, interaction with great apes, clothing, hygiene, anthelmintic treatment and dietary habits. All participants spoke French and researchers assisted them to fill in the questionnaires.
(JPG)

S1 Table. Accession numbers (from the European Nucleotide Archive) and related metadata for each sample.
(XLSX)

Acknowledgments

The authors would like to express special thanks go to the Ministère de la Recherche Scientifique et de l'Innovation and Ministère des Forêts et de la Faune, Cameroon for permission to conduct the research in Cameroon; Antwerp Zoo Society, Belgium and Project Grands Singes, Cameroon for welcoming the project and logistical support in the field; and mainly to all local trackers, communities, assistants and other people, who helped with sample collection: Arlette Tchankugni Nguemfo, Klára Vlčková, Zuzana Tehlárová and Dagmar Jirsová. The authors acknowledge Core Facility Genomics within CEITEC, Masaryk University supported by the NCMG research infrastructure (LM2018132) for their support with obtaining scientific data presented in this paper. Computation resources were provided by the e-Infrastruktura CZ project (ID:90140), supported by the Ministry of Education, Youth and Sports of the Czech Republic.

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Vlčková, K., Kreisinger, J., Pafčo, B., Čížková, D., Tagg, N., Hehl, A.B., Modrý, D.

2018

Diversity of *Entamoeba* spp. in African great apes and humans: an insight from Illumina MiSeq high-throughput sequencing.

Int. J. Parasitol. 48, 229–239. https://doi.org/10.1016/j.ijpara.2017.11.008

International Journal for Parasitology 48 (2018) 519–530

Contents lists available at ScienceDirect

International Journal for Parasitology

journal homepage: www.elsevier.com/locate/ijpara

Diversity of *Entamoeba* spp. in African great apes and humans: an insight from Illumina MiSeq high-throughput sequencing

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ARTICLE INFO

Article history:
Received 16 August 2017
Received in revised form 2 November 2017
Accepted 7 November 2017
Available online 10 March 2018

Keywords:
Entamoeba
Western lowland gorilla
Central chimpanzee
Humans
Metabarcoding
Diversity
Mixed infections
Entamoeba histolytica

ABSTRACT

Understanding the complex *Entamoeba* communities in the mammalian intestine has been, to date, complicated by the lack of a suitable approach for molecular detection of multiple variants co-occurring in mixed infections. Here, we report on the application of a high throughput sequencing approach based on partial 18S rDNA using the Illumina MiSeq platform. We describe, to our knowledge, for the first time, the *Entamoeba* communities in humans, free-ranging western lowland gorillas and central chimpanzees living in the Dja Faunal Reserve in Cameroon. We detected 36 *Entamoeba* haplotypes belonging to six haplotype clusters, containing haplotypes possessing high and low host specificity. Most of the detected haplotypes belonged to commensal *Entamoeba*, however, the pathogenic species (*Entamoeba histolytica* and *Entamoeba nuttalli*) were also detected. We observed that some *Entamoeba* haplotypes are shared between humans and other hosts, indicating their zoonotic potential. The findings are important not only for understanding the epidemiology of amoebiasis in humans in rural African localities, but also in the context of wild great ape conservation.

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1. Introduction

Intestinal protists of the genus *Entamoeba* (Casagrandi and Barbagallo, 1895) mostly colonise the digestive system of a wide range of hosts from invertebrates to humans (Hooshyar et al., 2015). *Entamoeba* organisms have simple life cycles consisting of a vegetative stage, the trophozoite, which lives in the host's large intestine, and the cyst, which allows survival outside the host and transmission to a new host. Two species, namely *Entamoeba moshkovskii* (Tshalaia, 1941) and *Entamoeba ecuadoriensis* (Clark and Diamond, 1997) are generally considered to be primarily free-living organisms and can survive in trophozoite form outside the host. *Entamoeba gingivalis* (Gros, 1849), which colonises the oral cavity of a range of mammals, does not possess the ability to form cysts, instead of being transmitted directly in the trophozoite form (Duchêne, 2016).

The genus *Entamoeba* contains many species, seven of which (*E. histolytica* Schaudinn, 1903, *E. dispar* Brumpt, 1925, *E. nuttalli* Castellani, 1908, *E. moshkovskii*, *E. coli* Grassi, 1879, *E. poleckii* von Prowazek, 1912, *E. hartmanni* von Prowazek, 1912) are known to occur in non-human primates (NHPs) (Verweij et al., 2003b; Tachibana et al., 2009; Levecke et al., 2010; Jirků-Pomajbíková et al., 2016; Nolan et al., 2017). *Entamoeba nuttalli* and *E. histolytica* are recognised as pathogenic species. The latter is responsible for an estimated 35–50 million cases of symptomatic disease and approximately 100,000 deaths annually (WHO/PAHO/UNESCO, 1997), making human amoebiasis the third leading cause of human death due to parasitic disease (WHO, 1997). *Entamoeba nuttalli*, the species most closely related to *E. histolytica*, is a pathogen of macaques (*Macaca mulatta, Macaca sinica aurifrons*) (Tachibana et al., 2007, 2013; Feng et al., 2013), colobus monkeys (*Colobus guereza*) and spider monkeys

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https://doi.org/10.1016/j.ijpara.2017.11.008
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(*Ateles geoffroyi*) (Suzuki et al., 2007). The remaining species are usually non-pathogenic, however, *E. dispar* and *E. moshkovskii* may exhibit some pathogenicity in humans (Ali et al., 2003; Tengku and Norhayati, 2011; Serrano-Luna et al., 2013). *Entamoeba* spp. are commonly present in mixed infections (Verweij et al., 2003a; Rojas et al., 2016). Clinical diagnostics is usually performed by light microscopy (Hamzah et al., 2006) and species differentiation is based on cyst morphology, namely cyst size and the number and morphology of the nuclei. However, exact identification is complicated by the similarity between cysts of several species. For instance, to differentiate the cysts of *Entamoeba* with four nuclei (with the exception of *E. hartmanni* forming much smaller cysts) is impossible (Kebede et al., 2004) even though this group includes pathogenic (*E. histolytica* and *E. nuttalli*), non-pathogenic (*E. dispar*) and amphizoic (*E. moshkovskii*) species. Thus, molecular approaches are crucial for a differential diagnosis (Verweij et al., 2003a; Tachibana et al., 2007).

There are several studies which used molecular tools to detect *Entamoeba* spp. in primates, although the majority of these only examined captive primates and focused only on selected *Entamoeba* spp. (Verweij et al., 2003b; Tachibana et al., 2009; Levecke et al., 2010; Rivera et al., 2010; Regan et al., 2014). Some studies have adopted molecular tools for *Entamoeba* spp. detection in free-ranging primates, including only two studies conducted on great apes. PCR amplification of partial 18S rDNA was used to detect *Entamoeba* spp. in wild rhesus macaques (*Macaca mulatta*) (Tachibana et al., 2013; Guan et al., 2016; Debenham et al., 2017), wild Tibetan macaques (*Macaca thibetana*) (Guan et al., 2016), wild toque macaques (*Macaca sinica aurifrons*) (Tachibana et al., 2016), wild Celebes crested macaques (*Macaca nigra*) (Tuda et al., 2016), wild howler monkeys (*Alouatta palliata* and *A. pigra*) (Villanueva-García et al., 2017), and captive long-tailed macaques (*Macaca fascicularis*) and wild rhesus macaques (Feng et al., 2013). Jirků-Pomajbíková et al. (2016) used semi-nested PCR to detect *Entamoeba* in wild chimpanzees (*Pan troglodytes schweinfurthii*) from Issa Valley, Tanzania and conducted molecular-phylogenetic analyses based on partial 18S rDNA. Nolan et al. (2017) detected *E. coli* and *E. hartmanni* in habituated mountain gorillas based on partial 18S rDNA.

Application of classical PCR-based methods followed by Sanger sequencing is, to some extent, limited by the common occurrence of more than one species/genotype. High throughput sequencing (HTS) techniques provide a useful approach for overcoming such difficulties. Pyrosequencing has been used to detect *Entamoeba* spp. in humans (Stensvold et al., 2010). In our study, we developed an innovative HTS approach based on the Illumina MiSeq platform, targeting part of the 18S rDNA to describe diversity and host specificity of *Entamoeba* spp. in sympatric primates, namely humans, western lowland gorillas, and central chimpanzees on the periphery of the Dja Faunal Reserve, Cameroon. The approach developed represents a useful solution for detection of co-infection of a single host by several *Entamoeba* spp./haplotypes.

2. Materials and methods

2.1. Study site and sample collection

Faecal samples from free-ranging central chimpanzees (*Pan troglodytes troglodytes* Blumenbach, 1775) (n = 23), western lowland gorillas *Gorilla gorilla gorilla* Savage and Wyman, 1847) (n = 35), and humans (n = 132) living in close proximity were collected from September to October 2014 on the periphery of the Dja Faunal Reserve in Cameroon (Fig. 1). The samples were collected non-invasively early in the morning from gorilla night nests and on the forest floor underneath chimpanzee night nests only, to avoid re-sampling of the same individuals, and only from fresh nests sites, thus ensuring only fresh faeces were obtained i.e. within 3 h from the time we suspected the gorillas and chimpanzees to have left the nests. The samples were collected from within the core of the faeces and placed in collection tubes containing 96% ethanol. To avoid repeated sampling of the same groups, we either sampled groups of significantly different size (based on the number of nests in the nest sites) within the same area or groups of approximately the same size only if they were sufficiently spatially separated. The

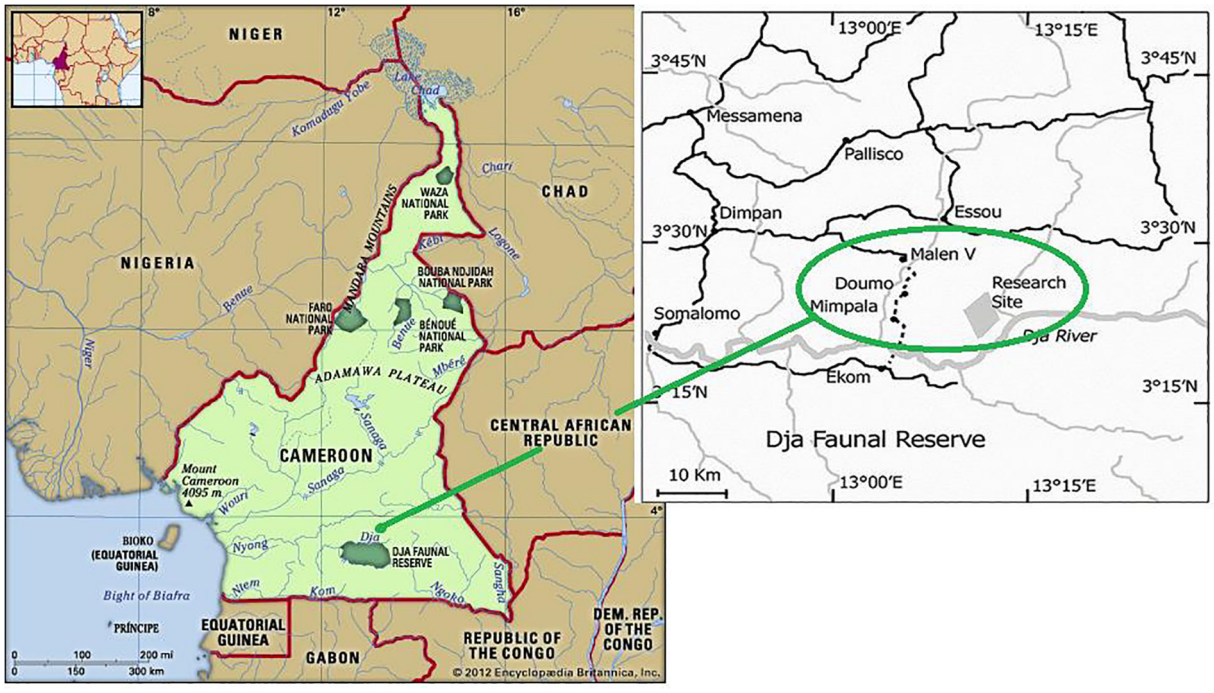


Fig. 1. Map of the study site in Cameroon. (Map adapted from Encyclopaedia Britannica, Inc. and Isra et al., 2007.)

human samples were collected in three villages: Duomo-pierre, Malen V, and Mimpala. The collection protocol was approved by an ethics committee (Ethical Commission of the Biology Centre of the Academy of Sciences, České Budějovice, Czech Republic) and prior informed consent was obtained from all volunteers. Preserved samples were kept at room temperature for a maximum of 2 months before transport to the Department of Pathology and Parasitology, University of Veterinary and Pharmaceutical Sciences Brno (UVPS), Czech Republic, where those were kept at −20 °C until DNA was extracted.

2.2. Molecular analyses and data processing

PrimerProspector (Walters et al., 2011) was used to design primers that specifically amplified *Entamoeba* 18S rDNA. The Silva database (version 128; Quast et al., 2013) for eukaryotic 18S rDNA was used as a reference for primer design and for subsequent *in silico* evaluation of their specificity. We also verified the suitability of resulting primers using the online TestPrime tool (Klindworth et al., 2013). Results of these *in silico* procedures indicated that primers used in this study capture all known *Entamoeba* 18S rDNA haplotypes, whereas amplification of non-target taxa was negligible. The primers were designed on the highly-conserved flanking regions and targeted the *Entamoeba* hypervariable region. DNA from faecal samples was extracted using the Power Soil DNA Isolation Kit (MoBio Laboratories Inc., Carlsbad, CA, USA), per the manufacturer's protocol. To remove ethanol from samples prior to DNA extraction, each sample was dried overnight at 37 °C. DNA from known *Entamoeba* spp. previously detected in primates, including *Entamoeba muris* due to its close phylogenetic relationship with *E. coli*, obtained from xenic and axenic cultures, was used as a positive control to optimise the PCR approach. PCR and primer optimization was done at the Institute of Parasitology, University of Zurich, Switzerland. Sanger sequences of these samples (sequenced at Microsynth AG, Balgach, Switzerland) were used as references in later analyses and the identities of obtained sequences were confirmed by BLASTn searches against the nt/nr NCBI database (GenBank), Table 1.

An approximately 270 bp long region of 18S rDNA was amplified with two-round PCR. The first PCR round was performed (94 °C for 30 s, 35 cycles at 98 °C for 20 s, 55 °C for 15 s, 72 °C for 15 s, and 72 °C for 1 min) using our designed *Entamoeba*-specific primers 673f (5′-ATYAGATACCGTCGTAGTCC-3′) and 942r (5′-GTWC GGTCTTGGTAAGTTTTC-3′) with overhangs comprised of Illumina sequencing sites for the second PCR. The PCR mixture contained 1 μl of 10× diluted DNA, 5 μl of Phusion Hot Start II High-Fidelity PCR Master Mix (Thermo Fisher Scientific, USA), 0.4 μl of each primer (10 μM), and 3.2 μl of PCR H_2O. The second PCR round was performed (95 °C for 3 s, 15 cycles at 98 °C for 20 s, 55 °C for 15 s, 72 °C for 15 s, and 72 °C for 1 min) with a 25× diluted PCR product from first PCR round using outer PCR primers containing sample-specific barcodes and sequencing adaptors (Access Array Barcode Library for Illumina Sequencers, Fluidigm Corporation, USA). The PCR mixture contained 2 μl of 25× diluted PCR product from first PCR round, 5 μl of KAPA HiFi HotStart ReadyMix PCR Kit (Kapa Biosystems, USA), and 3 μl of the outer PCR primers (50 μM). Amplicon purification was performed using Agencourt AMPure XP beads (Beckman Coulter, California, USA) and Pippin Prep (Sage Science, Massachusetts, USA). Amplicon sequencing was performed using a MiSeq Reagent Kit v2 (2 × 250 bp paired-end reads) in the Illumina MiSeq Platform. The HTS library preparation and sequencing analyses were performed at the Central European Institute of Technology (CEITEC), Masaryk University, Brno, Czech Republic.

Primers were trimmed using skewer (Jiang et al., 2014). Subsequently, using dada2 (Callahan et al., 2016) we eliminated sequences where the expected number of sequencing errors was higher than one and performed denoising on the filtered dataset to estimate relative abundances of 18S rDNA haplotypes in individual samples. Scripts associated with these bioinformatic steps are provided in Supplementary Data S1. According to our previous analyses, the default dada2 algorithm for chimaera detection is associated with a considerable risk of false positives (i.e. real 18S rDNA haplotypes being erroneously identified as chimaera, Pafčo et al., unpublished data). Instead, we used stringent filtering of the resulting community table to remove putatively chimeric sequences and other spurious 18S rDNA haplotypes. We eliminated from each sample all haplotypes that were represented by <4% sequences. In addition, assuming that the probability of independent formation of the same chimeric variant is low, we did not consider any 18S rDNA haplotypes that were present in just a single sample. Our previous benchmark tests aimed at the sequencing of "mock" communities of known composition, using nearly identical wet lab procedures and data processing (Pafčo et al., unpublished data), suggest that our pipeline is sufficiently robust against inflation of observed haplotype diversity due to unfiltered PCR and/or sequencing artefacts.

Taxonomic assignment of the resulting 18S rDNA haplotypes was performed using (i) BLASTn searches against the nt/nr database, (ii) phylogenetic analyses, where a custom database of 18S rDNA sequences was used as a reference and (iii) phylogenetic analyses, where a custom database of 18S rDNA sequences and NCBI records corresponding to *Entamoeba* 18S rDNA were used as a reference. Sequence alignment was performed using DECIPHER algorithms (Wright, 2016) that account for the secondary structure of rRNA. Maximum likelihood (ML) phylogenetic trees were constructed using RAxML and bootstrapping analyses ($n = 1000$ replicates) were subsequently used to assess their

Table 1
Reference material: *Entamoeba* spp. isolates previously detected in primates, used as reference sequences in analyses of the high-throughput sequencing dataset. *Entamoeba muris* is included due to its close phylogenetic relationship with *Entamoeba coli*.

Entamoeba species	Source of DNA	GenBank acc. No.	Nearest hit in GenBank (97% identity)
E. hartmanni DMP07	Xenic culture	MF631986	Sequence ID: FR686380.1 (100%)
E. moshkovskii (soil)	Xenic culture	MF631987	Sequence ID: KT825987.1 (97%)
E. moshkovskii Laredo	Axenic culture	MF631988	Sequence ID: KT825989.1 (100%)
E. bangladeshi 8111	Xenic culture	MF631989	Sequence ID: KR025411.1 (100%)
E. dispar SAW760	Axenic culture	MF631990	Sequence ID: KT825981.1 (100%)
E. nuttalli 360	Xenic culture	MF631991	Sequence ID: AB426549.1 (100%)
E. histolytica HM-1 IMSS	Xenic culture	MF631992	Sequence ID: GQ423749.1 (100%)
E. histolytica HM-1 IMSS	Axenic culture	MF631993	Sequence ID: GQ423749.1 (100%)
E. histolytica Rahman	Axenic culture	MF631994	Sequence ID: GQ423749.1 (100%)
E. histolytica 2592100	Axenic culture	MF631995	Sequence ID: GQ423749.1 (100%)
E. coli ST1 (human)	Xenic culture	MF631996	Sequence ID: AB444953.1 (99%)
E. coli ST2 (gorilla)	Xenic culture	MF631997	Sequence ID: FR686434.1 (99%)
E. muris	Xenic culture	MF631998	Sequence ID: AB445018.1 (99%)

integrity. Phylogeny, together with abundance patterns of 18S rDNA variants in individual samples, were visualised using the ggtree package (Yu et al., 2017) in R v. 3.1.0 (R Core Team, 2014, Vienna, Austria).

Changes in the diversity of 18S rDNA haplotypes were assessed using generalised linear models (GLMs) with Poisson error distribution. Among-sample differentiation in relative abundances of 18S rDNA variants was visualised using Principal Coordinate Analysis (PCoA) for Bray-Curtis dissimilarities. We further used a negative GLM framework for multivariate data implemented in the mvabund package to test for systematic differences in *Entamoeba* profiles among host species and to identify 18S rDNA variants driving these inter-host changes (Wang et al., 2012). All statistical analyses were performed in R. $P = 0.05$ was considered statistically significant.

2.3. Data accessibility

A Phyloseq object containing operational taxonomic unit (OTU) tables, sample metadata, haplotype sequences and their taxonomic assignations are available at Mendeley Data (doi: 10.17632/pmxksr57wn.1). Raw sequencing files were submitted to the European Nucleotide Archive (ENA) and are publically accessible under the following link: http://www.ebi.ac.uk/ena/data/view/PRJEB23126. ENA accession numbers for individual samples are included in the Phyloseq object.

3. Results

3.1. Overall diversity of Entamoeba haplotypes

The resulting dataset, after all filtering steps, included 3,524,128 high-quality sequences that were distributed across 36 18S rDNA haplotypes (Fig. 2). According to BLASTn searches, all haplotypes corresponded to *Entamoeba* spp. We grouped the detected haplotypes (OTUs) into six putatively monophyletic clusters termed haplotype groups (*E. hartmanni*, *E. dispar*, *E. nuttalli*, *E. histolytica*, *E. invadens* and *E. coli*), according to their phylogenetic relatedness to reference sequences (Table 2, Supplementary Fig. S1). The most numerous haplotypes were those related to the *E. coli* and *E. hartmanni* reference sequences and a haplotype identical to *E. dispar* SAW760. To further assess the variability within the most haplotype-rich *E. coli* and *E. hartmanni* haplotype groups, we performed phylogenetic analyses that included

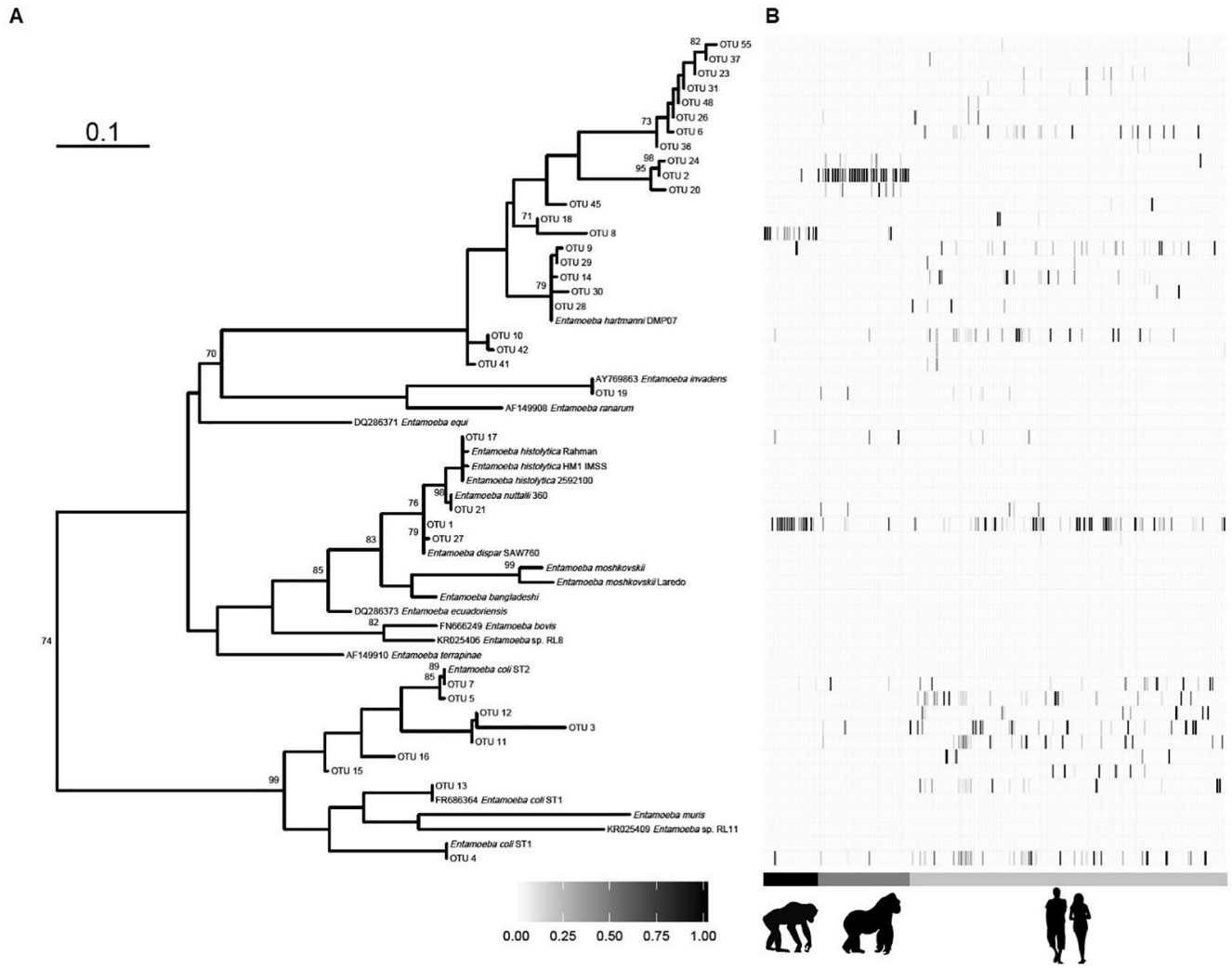


Fig. 2. *Entamoeba* haplotypes detected in faecal samples of studied host species (central chimpanzees, western lowland gorillas, and humans). (A) Maximum likelihood phylogeny for *Entamoeba* 18S rDNA sequences. Sequences detected in faecal samples are labelled as operational taxonomic units, haplotypes gathered from cultures using Sanger sequencing are labelled as certain *Entamoeba* spp. and sequences obtained from GenBank are labelled with accession numbers. (B) Heatmap showing representation of haplotypes in individual samples. *Endolimax nana* was used as a root. Bootstrap values below 70% are not shown.

Table 2
The divergence (% of nucleotides) of sequences of detected haplotypes (~270 bp) grouped into six putatively monophyletic clusters termed as haplotype groups (*Entamoeba hartmanni*, *Entamoeba dispar*, *Entamoeba nuttalli*, *Entamoeba histolytica*, *Entamoeba invadens*, and *Entamoeba coli*), according to their phylogenetic relatedness to reference samples (obtained from xenic and axenic cultures) with corresponding reference sequences from NCBI database.

Reference sample	Haplotype	Divergence (% of nt)	GenBank acc. No.
Entamoeba hartmanni DMP07	OTU 28	0.6	MG256541
	OTU 29	1.2	MG256542
	OTU 14	1.2	MG256529
	OTU 9	1.8	MG256524
	OTU 30	2.4	MG256543
	OTU 18	7.2	MG256533
	OTU 8	8.4	MG256523
	OTU 41	8.4	MG256547
	OTU 45	8.4	MG256549
	OTU 10	9.0	MG256525
	OTU 42	9.0	MG256548
	OTU 23	12.0	MG256537
	OTU 37	12.0	MG256546
	OTU 36	12.6	MG256545
	OTU 55	12.6	MG256551
	OTU 26	13.2	MG256539
	OTU 31	13.2	MG256544
	OTU 48	13.2	MG256550
	OTU 6	13.8	MG256521
	OTU 2	15.6	MG256517
	OTU 20	16.2	MG256535
	OTU 24	16.2	MG256538
Entamoeba dispar SAW760	OTU1	0	MG256516
	OTU 27	0.4	MG256540
Entamoeba nuttalli 360	OTU 21	0	MG256536
Entamoeba histolytica Rahman/HM1 IMSS/2592100	OTU 17	0	MG256532
Entamoeba invadens	OTU 19	7.2	MG256534
Entamoeba coli ST1	OTU 4	10.8	MG256519
	OTU 13	19.3	MG256528
E. coli ST2	OTU 16	9.00	MG256531
	OTU 3	14.8	MG256518
	OTU 12	11.0	MG256527
	OTU 5	9.5	MG256520
	OTU 7	10.5	MG256522
	OTU 15	9.0	MG256530
	OTU 11	11.0	MG256526

OTU, operational taxonomic unit.

available sequences from GenBank (indexed as *E. hartmanni* or *E. coli*) and confirmed their relatedness (Figs. 3 and 4). *Entamoeba histolytica* and *E. nuttalli* occurred as a single haplotype each, identical to reference sequences of *E. histolytica* Rahman/2592100/HM-1 IMSS strains and *E. nuttalli* 360 strain, respectively. A haplotype distantly related to *E. invadens* was detected in a few samples. No sequences exhibiting considerable relatedness to *E. moshkovskii*, *E. bangladeshi* (Royer, Gilchrist, Kabir, Arju, Ralston, Haque, Clark & Petri, 2012) or *E. poleckii* were detected (Fig. 2, Supplementary Fig. S1). Sequence divergences between the detected haplotypes and corresponding reference samples are listed in Table 2.

3.2. Mixed infections

Mixed infections (the presence of more than one haplotype) were revealed in 63% of samples, and the average number of haplotypes detected in individual samples was 2.27 (median = 2, interquartile range = 1–7). Within-sample diversity of haplotypes in individual host species is shown in Figs. 5A, 6; all mixed infections also are obvious from Supplementary Fig. S2. Haplotypes from more than one *Entamoeba* haplotype group were present in 43% of samples. The representation of individual haplotype groups across examined samples is shown in Fig. 5B.

3.3. Entamoeba communities in examined host species

We found 33 haplotypes belonging to six haplotype groups in humans, 14 haplotypes belonging to six haplotype groups were detected in gorillas, and eight haplotypes belonging to four haplotype groups in chimpanzees (Fig. 7). Haplotype diversity varied among host species (Fig. 6; quasi-Poisson GLM: Δ D:F: = 2, $\chi^2 = 14.512$, $P < 0.001$). Whereas there was no difference in haplotype diversity among gorillas and chimpanzees according to Tukey post hoc tests ($P = 0.9$), the haplotype diversity in humans was significantly higher than that of chimpanzees and gorillas ($P < 0.05$). PCoA and mvabund analyses indicated pronounced differentiation of *Entamoeba* profiles among different hosts (Figs. 8 and 9).

The spectrum of OTUs in the examined host species and numbers of infected individuals is shown in Fig. 7 and Supplementary Table S1. In chimpanzees, there were two common haplotypes, namely OTU 1 from the *E. dispar* haplotype group found in 15 individuals and OTU 8 from the *E. hartmanni* haplotype group present in 13 individuals. Another five detected haplotypes (OTU 2, OTU 10, OTU 17, OTU 7, OTU 4) in chimpanzees were quite rare, captured only in one individual and in the case of OTU 7 in two individual chimpanzees. Three haplotypes (OTU 2, OTU 24, OTU 20) belonging to the *E. hartmanni* haplotype group were the most common in gorillas and clustered into a gorilla-specific clade within the *E. hartmanni* haplotype group (Fig. 7). Another 11 haplotypes were detected in no more than three gorillas. In humans, OTU 1 belonging to the *E. dispar* haplotype group was the most abundant haplotype. Seventeen haplotypes within the *E. coli*, *E. dispar*, *E. invadens* and *E. hartmanni* haplotype groups were present in more than three human samples; 15 haplotypes from all *Entamoeba* haplotype groups, with the exception of the *E. coli* haplotype group, were detected in no more than three humans. Sampled humans originated from three villages. The individual OTUs and number of infected humans are shown in Fig. 10. Although PCoA ordination did not indicate any clear clustering of human samples according to the sample location (village) (Supplementary Fig. S3), mvabund analyses revealed significant spatial variation in the composition of *Entamoeba* profiles (Δ D:F: = 2, deviance change = 123.634, $P = 0.001$). The univariate mvabund test suggests that this variation was driven by an increase in abundance of OTU 15 ($P = 0.063$) in Duomo Pierre and of OTU 19 in Malen V ($P = 0.034$, Supplementary Fig. S4).

4. Discussion

The limitations of commonly used methods, e.g. classical coproscopy, PCR and Sanger sequencing, warrant the application of advanced diagnostic approaches for *Entamoeba* detection in samples containing multiple species/genotypes (mixed infections). Multiplex PCR with a mix of species-specific primers (Zebardast et al., 2014) or a reverse line blot method using specific probes (Verweij et al., 2003a) resolve, to some extent, the problem of identifying mixed infections. Nevertheless, the *a priori* knowledge of targeted species is necessary for these methods and they lack sensitivity to detect different genotypes within *Entamoeba* spp. On the contrary, the use of carefully designed universal *Entamoeba* primers represents an unbiased approach. Cloning of PCR products can be used to identify individual species/genotypes (Fotedar et al., 2007); however, capturing rare genotypes is unlikely and this method is time-consuming and costly. The HTS approach is a superior method to effectively detect individual members of *Entamoeba* communities, including rare and unknown species, and to enable the processing of numerous samples. Here, we developed a HTS approach targeted at *Entamoeba* spp. using the Illumina MiSeq platform. The approach, based on *Entamoeba* spp. universal

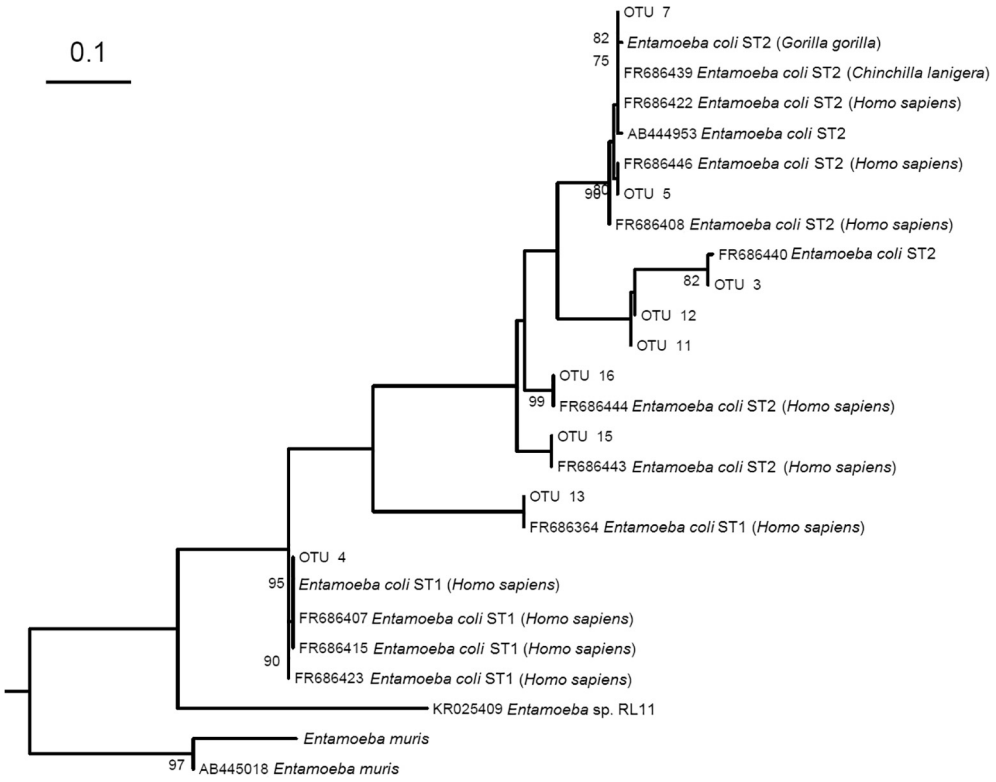


Fig. 3. Maximum likelihood phylogeny for 18S rDNA sequences from the *Entamoeba coli* haplotype group detected in faecal samples (labelled as operational taxonomic units), from haplotypes gathered from cultures using Sanger sequencing (*E. coli* ST1, *E. coli* ST2) and from sequences obtained from GenBank (labelled with accession numbers). Four sequences of *Entamoeba polecki* (Accession Nos. LC082304, LC082305, FR686392, FR686398) were used as an outgroup. Bootstrap values below 70% are not shown.

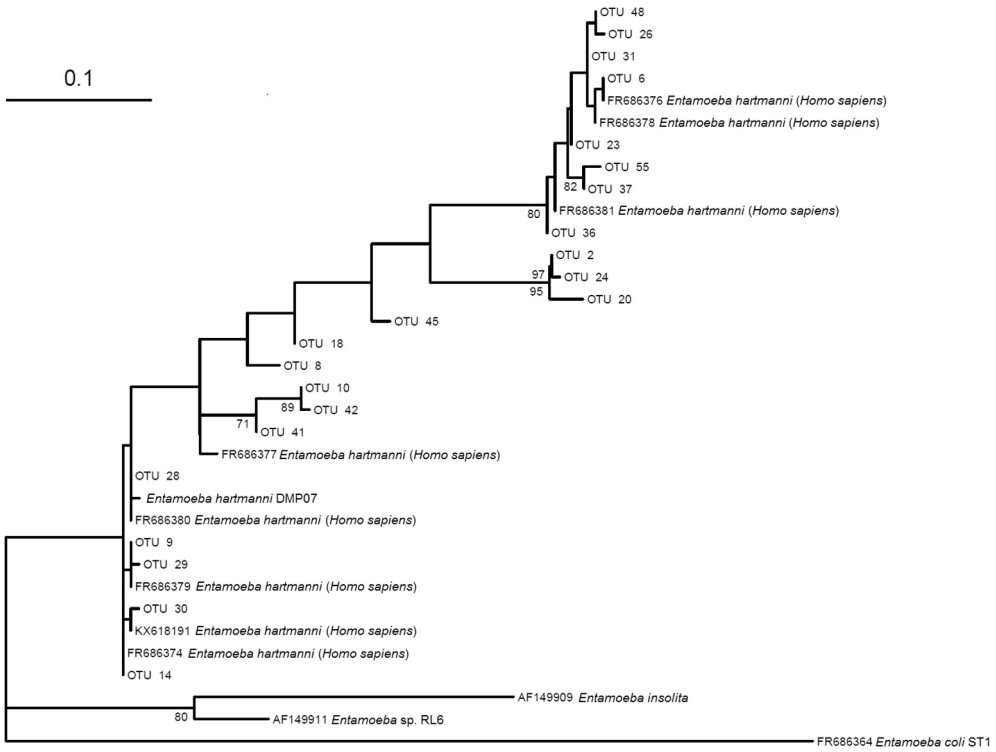


Fig. 4. Maximum likelihood phylogeny for 18S rDNA sequences from the *Entamoeba hartmanni* haplotype group detected in faecal samples (labelled as operational taxonomic units), from haplotypes gathered from cultures using Sanger sequencing (*E. hartmanni* DMP07) and from GenBank (labelled with accession numbers). Bootstrap values below 70% are not shown.

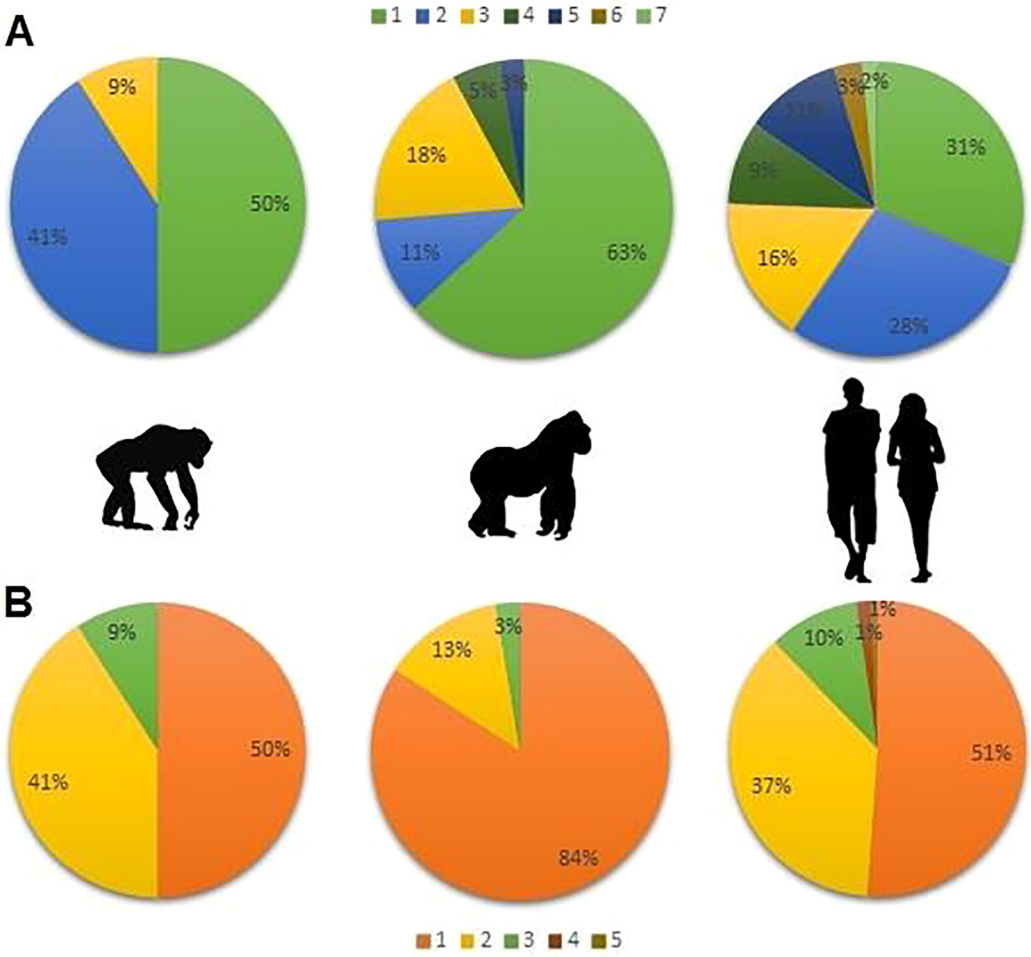


Fig. 5. *Entamoeba* haplotype mixed infections (A) Proportions of *Entamoeba* halotypes identified in individual samples according to host species (central chimpanzees, western lowland gorillas, and humans). (B) Number of haplotypes co-occurring in individual samples according to host species.

primers, effectively resolves *Entamoeba* mixed infections and captures rare species/genotypes such as OTU 19 which is distinct from, yet most closely related to, *E. invadens*.

From several potentially suitable markers (e.g. 18S rRNA, ITS, 5.8S rRNA) we selected the 18S rRNA gene, which is the most widely used marker in studies addressing the phylogenetic relationships among *Entamoeba* spp. (Verweij et al., 2001; Stensvold et al., 2010, 2011; Jirků-Pomajbíková et al., 2016; Tuda et al., 2016). Sequences of 18S rDNA contain highly conserved flanking regions suitable for the design of the universal primers. This genetic locus has the added benefit of occurring in multiple copies which facilitates PCR amplification, especially in the case of a low infection level. At the same time, 18S rDNA copies in *Entamoeba* spp. do not show sequence variation within a single genome (Bhattacharya et al., 1998) and multiple 18S rDNA variants were not detected by Sanger sequencing of the reference *Entamoeba* cultures.

Here, to our knowledge, we provide the first account of the *Entamoeba* community in free-ranging great apes (chimpanzees and gorillas) and humans living in close proximity on the periphery of the Dja Faunal Reserve in Cameroon. We describe the diversity of *Entamoeba* spp./haplotypes, their host specificity, and co-infections. Thirty-six *Entamoeba* haplotypes were detected, belonging to the six haplotype groups: *E. hartmanni*, *E. dispar*, *E. nuttalli*, *E. histolytica*, *E. invadens* and *E. coli*. The highest haplotype variability appeared in the *E. coli* and *E. hartmanni* haplotype groups. High variability within the *E. hartmanni* haplotype group corresponds

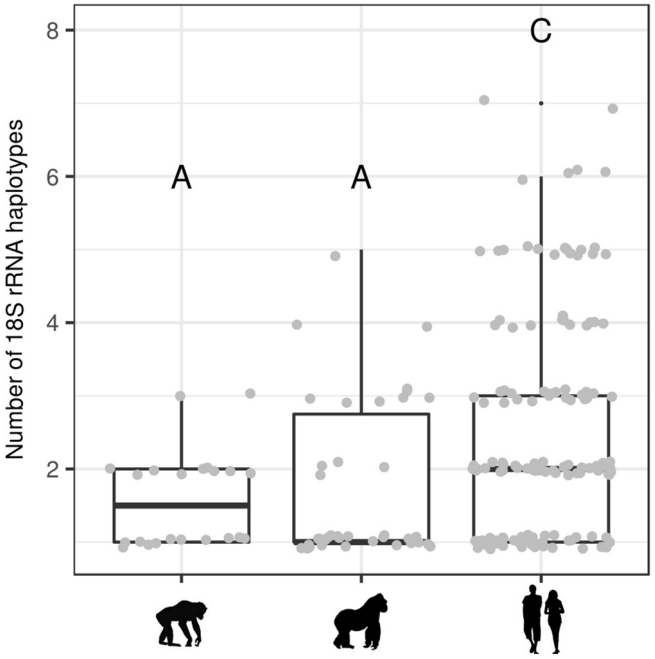


Fig. 6. Boxplot of number of *Entamoeba* haplotypes detected in individual host species (central chimpanzees, western lowland gorillas, and humans). Different letters above boxes indicate significant differences ($P < 0.05$ according to Tukey post hoc tests).

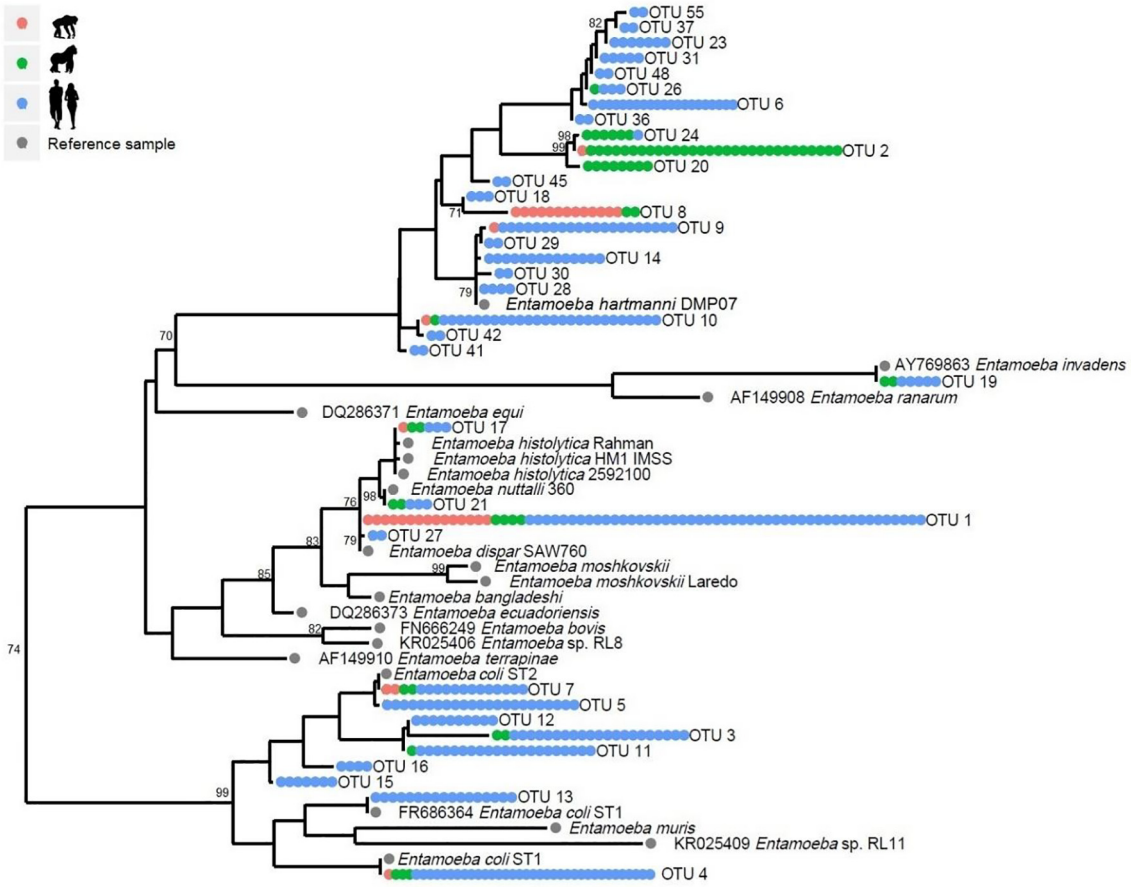


Fig. 7. Host spectrum (central chimpanzees, western lowland gorillas, and humans) and numbers of infected individuals shown for all detected *Entamoeba* haplotypes labelled as operational taxonomic units, haplotypes gathered from cultures using Sanger sequencing are labelled as certain *Entamoeba* spp. and sequences obtained from GenBank are labelled with accession numbers.

to that observed in chimpanzees in Tanzania (Jirků-Pomajbíková et al., 2016) and to previous findings reported in humans and other NHPs (Clark and Diamond, 1997; Stensvold et al., 2011). Comparatively, high variability has been observed within *E. coli* (Stensvold et al., 2011; Jirků-Pomajbíková et al., 2016). Contrary to *E. hartmanni*, however, *E. coli* haplotypes were closely related to already described variants, namely ST1 and ST2 subtypes, previously suggested as distinct species by Stensvold et al. (2011). *Entamoeba histolytica* and *E. nuttalli* were represented by one haplotype each, both having 100% identity with corresponding reference samples. *Entamoeba* OTU 19, the most related to *E. invadens* (GenBank accession No. AY769863), was repeatedly detected in gorillas and humans. We may assume this haplotype to be a putatively new species since the nucleotide divergence between these two sequences is more than 7%. *Entamoeba invadens* has been reported only in reptiles (Niculescu, 2015) and there has not been any evidence for its presence in mammalian hosts.

Importantly, the HTS approach allowed us to resolve the PCR amplicons from co-infecting *Entamoeba* haplotypes that are morphologically indistinguishable. The majority of detected *Entamoeba* haplotypes are phylogenetically related to those typically producing tetranucleated cysts with the exception of the octonucleated cyst-producing *E. coli*. We detected tetranucleated *Entamoeba* haplotypes in 155 samples, of which 70 contained more than two haplotypes and 43 contained haplotypes from more than two *Entamoeba* haplotype groups. Theoretically, therefore, more than 80% of samples from our dataset may be classified as *E. histolytica/dispar*-positive when adhering to the "classical" determination approach. This is in stark contrast with the fact that only six (3.2%) and five (2.6%) samples truly contained *E. histolytica* and *E. nuttalli* haplotypes, respectively. Previous microscopy-based studies detecting the tetranucleated cysts have presumed the occurrence of pathogenic *Entamoeba* in free-ranging great apes (Sleeman et al., 2000; Lilly et al., 2002), implying possible cross-transmission between humans and NHPs. Our results showed that pathogenic *Entamoeba* spp. important for human health are comparatively rare and the majority of tetranucleated *Entamoeba* spp. cysts more likely belong to the commensal species such as *E. dispar* and *E. hartmanni*.

The co-occurrence of the octo and tetranucleated *Entamoeba* cysts is common in free-ranging great apes as confirmed by coproscopic analysis (Gillespie et al., 2010; Petrášová et al., 2010; Kalousová, 2013, Gastrointestinal parasites of wild western lowland gorillas (*Gorilla gorilla gorilla*) in Dzanga Sector of Dzanga-Ndoki National Park, Central African Republic. Masters thesis, Masaryk University, Czech Republic), with investigators usually reporting the presence of unclassified tetranucleate *Entamoeba* spp. and/or *E. coli* (distinguishable by their octonucleated cysts). Some studies on chimpanzees and gorillas provide a more finite classification of tetranucleated *Entamoeba* cysts, reporting those as *E. hartmanni*, *E. histolytica* or *E. histolytica/dispar* (Sleeman et al., 2000; Lilly et al., 2002; Muehlenbein, 2005; Gillespie et al., 2010). However, considering the diversity of tetranucleated *Entamoeba* haplotypes and the regular occurrence of mixed infections as demonstrated by our study, all studies using microscopy to identify these *Entamoeba* spp. (except distinguishable small cysts of *E. hartmanni*) in faeces

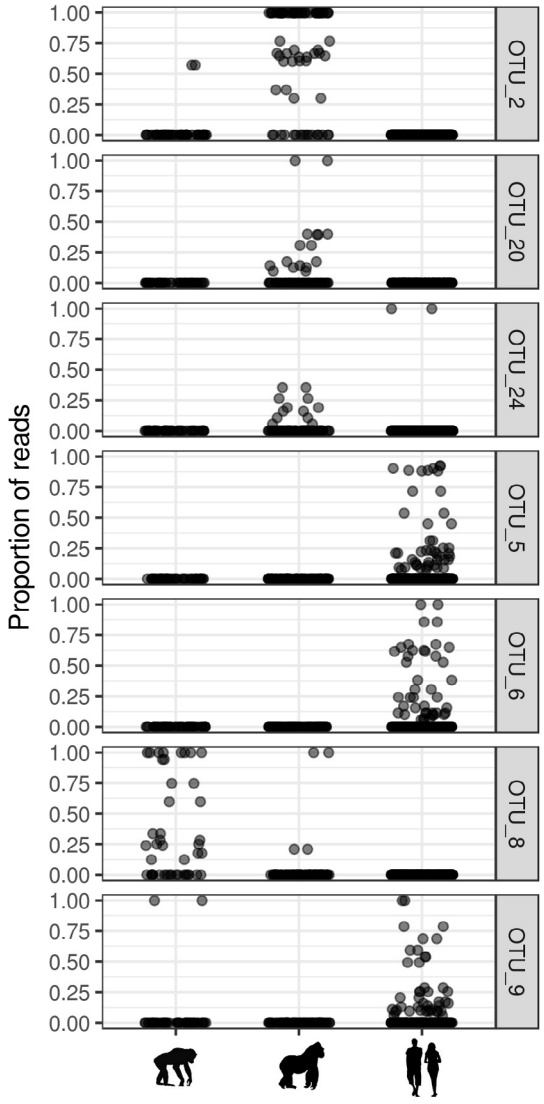


Fig. 8. Proportion of reads for *Entamoeba* 18S rDNA haplotypes, whose abundances varied among individual host species (central chimpanzees, western lowland gorillas, and humans) according to likelihood ratio test for mvabund models. OTU = operational taxonomic unit.

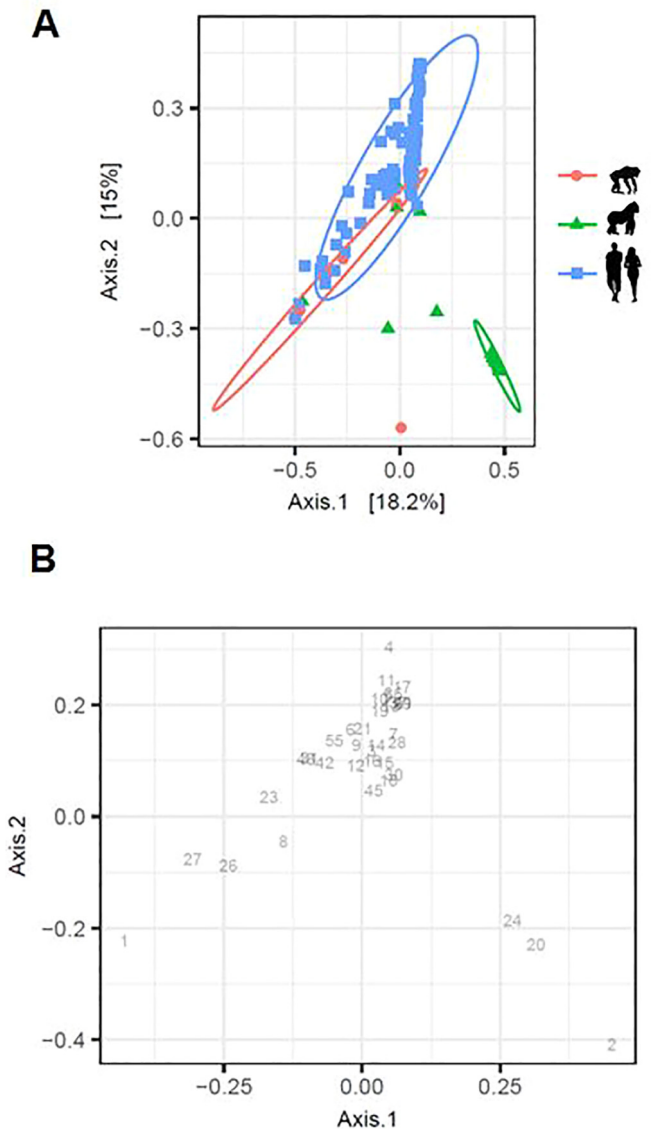


Fig. 9. Principal Coordinate Analysis ordination for Bray-Curtis dissimilarities of *Entamoeba* profiles. (A) Among-sample differentiation. Host species (central chimpanzees, western lowland gorillas, and humans) identity is indicated by different colours (shades of grey); 95% standard ellipses are shown. (B) Represent contribution of individual haplotypes to the among-sample variation.

must be taken with caution (Clark and Diamond, 1997; Kebede et al., 2004).

Based on ordination analyses, we showed that all three host species harbour distinct *Entamoeba* communities (Figs. 2, 6). While gorillas form a clearly separated cluster, the composition of human and chimpanzee *Entamoeba* communities overlap, displaying a pattern that reflects the phylogenetic distance between the hosts. When focusing on the individual members of the *Entamoeba* communities (Fig. 7), it is obvious that there are several haplotypes that drive the distinction of the communities, i.e. they are frequently found in one host and rarely in the others. This includes OTU 2, OTU 20, and OTU 24 which form a separate clade in the *E. hartmanni* haplotype group and which almost exclusively occurred in gorillas. Interestingly, no *Entamoeba* sequences present in the NCBI database fall into this "gorilla clade". Consequently, these OTUs represent *Entamoeba* haplotypes with specificity towards gorillas that probably represent novel species. The haplotype most closely associated with chimpanzees (OTU 8) is a constituent of the *E. hartmanni* group and is not closely related to any known sequence. In humans, we found three host-specific haplotypes which belong to *E. hartmanni* (OTU 6, OTU 9) and *E. coli* (OTU 5) groups, and are identical or very similar in sequence to others sequenced from humans. There are many other haplotypes, particularly in the *E. hartmanni* and *E. coli* groups, which occurred only in humans, but given their lower abundances, they did not have any major influence on the distinction between the communities. Moreover, it is possible that these haplotypes may occur in other hosts but have not been sampled due their low abundances. In theory, the limits of parasite specificity are determined by two processes or "filters": (i) the encounter filter determines whether the parasite can ever find the host; it is affected by geography, habitat use, behaviour, the ecology of the host, etc. and (ii) the compatibility filter determines whether the parasite succeeds in infecting the host and reproducing once it makes contact with the host (Combes, 2001). The relatively high host specificity of some haplotypes is most probably explainable by the compatibility filter since the studied hosts live in the close proximity which creates opportunities for between-species transmission.

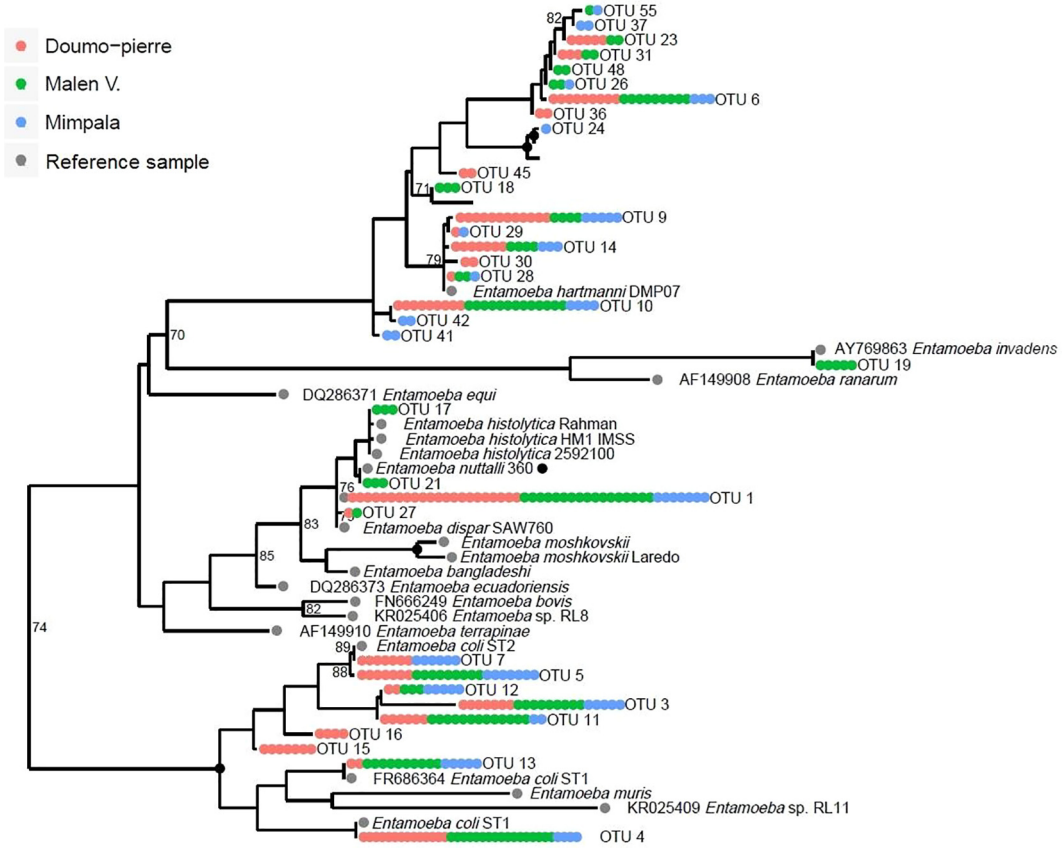


Fig. 10. All *Entamoeba* haplotypes detected in humans and numbers of infected individuals from three villages situated on the periphery of the Dja Faunal Reserve, Cameroon. *Entamoeba* haplotypes are labelled as operational taxonomic units, haplotypes gathered from cultures using Sanger sequencing are labelled as certain *Entamoeba* spp. and sequences obtained from GenBank are labelled with accession numbers.

Almost half of all detected haplotypes were found in two or all three host species. The major cause of the overlap between the communities is probably *E. dispar* haplotype OTU 1, which has the highest abundance of all the haplotypes and which was detected in many humans and chimpanzees, and in several gorillas. However, the haplotypes with lower host specificity also were present in the other haplotype groups, including those with potentially pathogenic species. The level of host specificity is of paramount importance, particularly in the case of pathogenic *E. histolytica*, especially the determination of its reservoirs and in evaluation of the extent of zoonotic infections in humans. The fact that the sequences of *E. histolytica* detected in our study were identical in NHPs and in humans, indicates its low host specificity and the zoonotic potential of this pathogenic species. However, further research based on multilocus data is required to verify this hypothesis, as we cannot exclude the possibility that there are host-specific strains corresponding to this particular OTU which we were not able to identify due to the low tempo of 18S rRNA evolution.

We detected two potentially pathogenic *Entamoeba* spp., namely *E. nuttalli* and *E. histolytica*. Although *E. histolytica* has been recorded in free-ranging great apes using light microscopy (Sleeman et al., 2000; Lilly et al., 2002; Gillespie et al., 2010), with all the above-mentioned limitations, this is the first time that its presence has been confirmed in free-ranging African great apes using DNA sequencing. *Entamoeba histolytica* is the cause of invasive amebiasis in humans (WHO, 1997) and experimentally in NHPs (Haq et al., 1985). However, the pathogenicity of *E. histolytica* seems to be associated with its strain/genotype (Escueta-de Cadiz et al., 2010; Kumari et al., 2013) which have been differentiated in some cases by variation in the numbers of short tandem repeats linked to their tRNA genes (Ali et al., 2008) or the genomic distribution of retrotransposable short interspersed nuclear elements (Kumari et al., 2013). The designed HTS approach does not allow *E. histolytica* strain identification which would require additional research. The pathogenicity of *E. histolytica* for great apes is poorly studied, despite similar pathologies to those of human invasive amebiasis having been reported in chimpanzees (Miller and Bray, 1966). However, the genetic factors underlying the pathogenicity of *E. histolytica* strains for great apes remain unknown.

Entamoeba nuttalli, the causative agent of invasive amebiasis in NHPs, was detected in both gorillas and humans in our study. Infections with this *Entamoeba* species may result in hemorrhagic dysentery, liver abscess or other extra-intestinal pathologies, even death (Loomis et al., 1983). *Entamoeba nuttalli* has previously been found in faecal samples of captive NHPs including chimpanzees and gorillas (Tachibana et al., 2009; Levecke et al., 2015) and, importantly, in animal caretakers working in a zoo where *E. nuttalli* infections were prevalent in animals (Levecke et al., 2015). The *E. nuttalli*-infected animal caretakers appeared to be completely asymptomatic, but in accordance with our findings, NHP-to-human transmission was assumed. A few studies have reported *E. nuttalli* in free-ranging NHPs, specifically in wild rhesus macaques and wild toque macaques (Tachibana et al., 2013, 2016). However, *E. histolytica* and *E. nuttalli* were only recently phylogenetically separated and thus their distribution is still poorly explored. To the best of our knowledge, this is the first study to report the detection of *E. nuttalli* in free-ranging great apes.

In conclusion, we developed an innovative approach for the analysis of *Entamoeba* communities based on *Entamoeba*-specific primers targeting partial 18S rDNA, sequenced using the Illumina

MiSeq platform. Using this method we described the *Entamoeba* communities occurring in humans and free-ranging African great apes and revealed their overlap, zoonotic potential, and host specificity. We also proved that the use of molecular methods is essential for species demarcation of *Entamoeba* spp., some of which are morphologically identical, yet represent genetically distinct groups. However, the involvement of other molecular markers for further identification of *E. histolytica* strains is necessary to ascertain their pathogenicity and the host preferences of certain *E. histolytica* genotypes. A better understanding of *Entamoeba* epidemiology and zoonotic potential in the rural African environment requires examination of additional host species including undomesticated animal species, rodents, livestock and other domestic animals. The HTS approach described here provides a powerful tool for such endeavours.

Acknowledgements

We thank the Ministère de la Recherche Scientifique et de l'Innovation a Ministère des Forêts et de la Faune, Cameroon for granting permission to conduct our research in Cameroon. We thank the Royal Zoological Society of Antwerp (KMDA), Belgium, Projet Grands Singes, Cameroon, and the World Wildlife Fund, Cameroon for logistical support in the field; Dagmar Jirsová, Zuzana Tehlarová, Arlette Tchankugni Nguemfo, Charmance Irene Nkombou and all local trackers and assistants for their help with sample collection. Special thanks go to Graham Clark, Seiki Kobayashi, Hiroshi Tachibana, Zuzana Hůzová, Ivan Čepička, and Ivona Foitová for providing *Entamoeba* cultures or DNA from *Entamoeba* cultures as reference samples to optimize molecular analyses. We thank Klára J. Petrželková from the Institute of Vertebrate Biology, Czech Academy of Sciences, Czech Republic, for her help with obtaining and coordinating the delivery of the reference samples. We thank Carmen Faso and Lenka Černíková from the Institute of Parasitology, University of Zurich, Switzerland, for their help and advice during PCR optimization and laboratory preparation. We acknowledge the Core Facility Genomics Central European Institute of Technology, Masaryk University supported by the National Center for Medical Genomics research infrastructure (LM2015091 funded by Ministerstvo Školství, Mládeže a Tělovýchovy, Czech Republic), especially Filip Pardy and Boris Tichý, for their support with obtaining scientific data presented in this paper. This publication derives from the Laboratory for Infectious Diseases Common to Humans and (non-Human) Primates, Czech Republic. This work was financially supported by the Internal Grant Agency of University of Veterinary and Pharmaceutical Sciences Brno, Czech Republic 138/2015/FVL, Internal Mobility Agency of University of Veterinary and Pharmaceutical Sciences Brno, Czech Republic 2015-FVL-19, Swiss European Mobility Programme, and co-financed by the project Central European Institute of Technology, Czech Republic (CZ.1.05/1.100/02.0068) from the European Regional Development Fund, allocated by the European Union.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.ijpara.2017.11.008.

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2024b

***Strongyloides* in non-human primates: significance for public health control.**

Philos. Trans. R. Soc. B 379, 20230006. https://doi.org/10.1098/rstb.2023.0006

PHILOSOPHICAL TRANSACTIONS B

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Review

Cite this article: Nosková E, Sambucci KM, Petrželková KJ, Červená B, Modrý D, Pafčo B. 2023 *Strongyloides* in non-human primates: significance for public health control. *Phil. Trans. R. Soc. B* **379**: 20230006. https://doi.org/10.1098/rstb.2023.0006

Received: 12 June 2023
Accepted: 18 September 2023

One contribution of 13 to a Theo Murphy meeting issue '*Strongyloides*: omics to worm-free populations'.

Subject Areas:
health and disease and epidemiology

Keywords:
Strongyloides, primates, microscopy, molecular methods

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Electronic supplementary material is available online at https://doi.org/10.6084/m9.figshare.c.6890521.

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Strongyloides in non-human primates: significance for public health control

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Primates are an important source of infectious disease in humans. Strongyloidiasis affects an estimated 600 million people worldwide, with a global distribution and hotspots of infection in tropical and subtropical regions. Recently added to the list of neglected tropical diseases, global attention has been demanded in the drive for its control. Through a literature review of *Strongyloides* in humans and non-human primates (NHP), we analysed the most common identification methods and gaps in knowledge about this nematode genus. The rise of molecular-based methods for *Strongyloides* detection is evident in both humans and NHP and provides an opportunity to analyse all data available from primates. Dogs were also included as an important host species of *Strongyloides* and a potential bridge host between humans and NHP. This review highlights the lack of molecular data across all hosts—humans, NHP and dogs—with the latter highly underrepresented in the database. Despite the cosmopolitan nature of *Strongyloides*, there are still large gaps in our knowledge for certain species when considering transmission and pathogenicity. We suggest that a unified approach to *Strongyloides* detection be taken, with an optimized, repeatable molecular-based method to improve our understanding of this parasitic infection.

This article is part of the Theo Murphy meeting issue '*Strongyloides*: omics to worm-free populations'.

1. Introduction

Due to their close phylogenetic relationship [1], pathogens are easily transmitted between humans and other primates, especially great apes [2]. Over 60% of infectious organisms known to be pathogenic to humans have an animal origin (are zoonotic) [3], and the origin of many human pathogens is in non-human primates (NHP). Although primates constitute a minority within the mammalian diversity, they are the original source for 20% of agents of human infectious diseases [4]. Notorious cases are among viruses, as major human viral diseases such as AIDS [5] and hepatitis B originate in NHP [6], and NHP also play a role as reservoirs for agents of other serious infections such as Ebola, yellow fever and monkeypox [7]. In addition to viruses, human parasites are evolutionarily linked to those in NHP. For example, *Plasmodium falciparum*, the most important parasite in humans [8], has been found to originate in the western lowland gorilla (*Gorilla gorilla gorilla*) [9]. On the other hand, human pathogens can have devastating effects on endangered populations of NHP. Respiratory diseases caused by pneumoviruses and paramyxoviruses [10], or tuberculosis [11] and measles [12] have been responsible for morbidities and mortalities of free-ranging and captive animals. While humans and NHP have always had some degree of contact in areas

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where they overlap [13], increased anthropogenic pressure from altered landscape use and subsistence activities [14] has resulted in an increased incidence of human–NHP contacts, consequently resulting in increased infection risk not only for humans but also for NHP populations [15].

Neglected parasites, including several soil-transmitted nematodes, are frequently transmitted between humans and NHP [16–19]. Rhabditid nematodes of the genus *Strongyloides* are among the most common soil-transmitted nematodes in both humans and NHP [16,20]. The genus *Strongyloides* includes over 50 species, of which three species are capable of establishing infection in humans. *Strongyloides stercoralis* has a cosmopolitan distribution and infects a broader range of hosts, including primates and carnivores [21]. Two lineages within *S. stercoralis* were established based on the molecular analyses: lineage A is probably zoonotic and occurs in humans, mainly in captive NHP and dogs, while the lineage B is probably restricted to dogs only [17,22]. In Africa and Asia, occasional infections by *Strongyloides fuelleborni* in humans are reported. The species was formally further classified into two subspecies. *Strongyloides f. fuelleborni* occurs in African and Asian NHP, with occasional transmission events to humans, while *S. f. kellyi* is endemic to Papua New Guinea (PNG) and infects humans only [23,24]. Although eggs of *Strongyloides* sp. have been found in human stool in the Indonesian part of New Guinea [25], they have never been proven to be from the same *Strongyloides* as in PNG, and furthermore, there is no report of it causing acute infantile disease as in PNG [23]. The classification of *S. f. kellyi* as a subspecies of *S. fuelleborni* was based on morphological and a separate isoenzyme electrophoretic result, which were not sufficiently different to warrant a specific distinction [26]. By contrast, the phylogenetic analyses did not group *S. f. kellyi* together with *S. f. fuelleborni*, suggesting that *S. f. kellyi* should be elevated to a species rank [27]. Adhering to this taxonomic concept, we use *S. fuelleborni* for infections in humans and NHP in Africa and Asia, with the exception of infections in humans in PNG, which are referred to as *S. kellyi*. Both *S. stercoralis* and *S. fuelleborni* are shared by humans and NHP, but there are more *Strongyloides* species recorded from NHP that have not yet been recorded in humans.

2. *Strongyloides* in non-human primates

Strongyloides infections are commonly reported in free-ranging and captive NHP on all continents except for Antarctica and Australia, although it is possible that captive primates in Australia are infected, and we are just missing the data. Unfortunately, a large proportion of studies report only the genus *Strongyloides* without specified species, often even without note for whether the observed stages were eggs or larvae. Undoubtedly, some of these findings are misidentified embryonated eggs of strongylid nematodes.

Strongyloides fuelleborni, first described by Von Linstow [28] in chimpanzees (*Pan troglodytes*) and yellow baboons (*Papio cynocephalus*) in Africa, is the most common *Strongyloides* species found in African and Asian primates (electronic supplementary material, table S1). *Strongyloides simiae* was described in Asian macaque (*Macaca* sp.) based on assumed morphological differences from *S. fuelleborni* [29]. However, only a couple of years later, it was concluded that the morphological traits of *S. simiae* are not sufficient for its recognition as a valid species [30,31]. Later authors further confirmed the conspecificity of *S. fuelleborni* and *S. simiae* [32,33]. Since then, *S. simiae* is considered a junior synonym of *S. fuelleborni*, which therefore remains the only oviparous species in Old Word NHP [33]. Analyses of the *cox1* sequences obtained from both African and Asian NHP show *S. fuelleborni* as a monophyletic, but quite highly diverse group [21]. At least two subclades—African and Indochinese peninsular clades—that may represent distinct species are distinguished [34]. Observed differences are rather connected to geographical distribution than to host species identity [21,35–37] and the geographically defined haplotypes can be shared among the sympatric primate species [38]. Historically, crossbreeding experiments with different strains of *Strongyloides* from chimpanzee, rhesus macaque (*Macaca mulatta*), tufted capuchin (*Sapajus apella*) and dog were performed [39]. No hybridization occurred, which led to the suggestion that strains from Asia, Africa and South America should be recognized as separate species. Nowadays, the South American *Strongyloides* is indeed treated as a separate species *S. cebus*. Nevertheless, Asian and African isolates are still referred to as a single species and discussion about the validity of *S. simiae* could be revisited as there is obviously a lack of gene flow between the distant geographical regions.

Strongyloides cebus has been identified only in NHP from the New World [27,40] and to date, there are no reports of human infection with this species. It was first described in capuchin monkeys (*Cebus capucinus*) [41] and redescribed based on the morphology of parasitic females [33]. In this species, ovaries are spiralled around the intestine and lips of the vulva are prominent, the tail tapers more gradually and is more sharply pointed than *S. fuelleborni* [33,40]. However, *S. cebus* differs also in the third-stage larvae (L3) and free-living adults' morphology and therefore, this species can be identified even without the necropsy of its host [40]. *Strongyloides cebus* was listed in several species of New World primates in America based on morphology of adults [40], although most studies from South America only report unidentified *Strongyloides* eggs or larvae when microscopic examination of faecal samples was conducted [42,43]. *Strongyloides cebus* was characterized genetically in an unspecified NHP from South America [27] and later in captive New World NHP in Japan [44] and the USA [45]. Phylogenetic analyses of mitochondrial sequences indicated *S. cebus* as a species rather distant from *S. fuelleborni* and *S. stercoralis*, suggesting that it evolved independently in the Americas as a parasite specific to NHP [44].

Interestingly, both *S. cebus* and *S. fuelleborni* are also found in captive primates in zoos, commonly outside of South America [44,45] or Africa [46]. Still, *S. fuelleborni* is rather uncommon in zoos, and its prevalence in captive or pet animals in countries of origin was always lower than in the free-ranging counterparts [47]. Although *S. cebus* produces eggs shed in the host faeces without capability for endogenous autoinfections, the larvae hatch shortly after defecation [40]. This species also differs by the number of generations in the free-living cycle; there are at least two generations in *S. cebus* [33], although three generations with fourth-generation eggs and larvae was described [48], while only one generation is typical for *S. fuelleborni* [33]. This could be responsible for its long persistence, potential of re-infection

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and accumulation of L3 in the environment [40], which may explain the persistence in zoos animals.

Strongyloides stercoralis is mainly diagnosed in captive settings [49,50], often related to unsanitary conditions facilitating transmission from humans or other reservoir hosts. *Strongyloides stercoralis* is the only ovoviviparous species among-primate infecting *Strongyloides*, with first-stage larvae (L1) capable of endogenous autoinfection. This ability, combined with the fact that NHP are kept in closed facilities, enables *S. stercoralis* infections to persist in captive NHP settings for prolonged periods. *Strongyloides stercoralis* was molecularly confirmed in captive chimpanzees [35], as well as captive Bornean orangutans (*Pongo pygmaeus*) and Sumatran orangutans (*Pongo abelii*) [50]. However, the presence of this species was reported also in Mahale Mountains National Park, Tanzania, in habituated free-ranging chimpanzees that were in contact with humans [35].

Molecular analyses revealed three cryptic and yet unnamed *Strongyloides* species in Bornean slow loris (*Nycticebus borneanus*) [38], spider monkeys (*Ateles geoffroyi*) [51] and in a colony of baboons (*Papio anubis*, *Papio hamadryas*) in a research institute in Texas [52]. The *cox1* sequences of *Strongyloides* from the loris clustered within the *S. stercoralis*/*procyonis* group [44,53], suggesting close phylogenetic relationships with these species. This clade also comprises a number of sequences of undetermined *Strongyloides* species found primarily in various hosts from the order Carnivora [54]. The *Strongyloides* sp. *cox1* sequences from spider monkeys clustered closely to the *S. papillosus*/*fuelleborni* group; a monophyletic group consisting of *S. fuelleborni* in primates but also ruminant-infecting *S. papillosus* and *S. vituli* as well as *S. venezuelensis* found in rats [44]. Finally, a short (366 bp) 18S rDNA sequence from the unspecified baboon clustered in a clade comprising *S. fuelleborni*, *S. stercoralis* and a *Strongyloides* sp. from a snake, while the second clade comprising *S. westeri*, *S. ratti*, *S. suis*, *S. venezuelensis*, *S. cebus*, *S. fuelleborni kellyi* and *S. papillosus* was formed [52].

3. Transmission of *Strongyloides* between non-human primates and humans

Cases of *S. fuelleborni* infection in humans have been reported mainly in the tropics, exclusively in areas where humans share their habitat with NHP. Although human infections with *S. fuelleborni* have been detected in both Africa and Asia, most reports are from tropical Africa (electronic supplementary material, table S1). *Strongyloides fuelleborni* has been found in numerous ethnic groups, mainly in rainforest areas [55] with the highest prevalence found in hunter–gatherers. A *S. fuelleborni* prevalence of 25% was reported among Babinga hunter–gatherers in the Central African Republic, with children being the most commonly infected [56]. High prevalence (49%) was found also in the Bambuti hunter–gatherers in the Democratic Republic of the Congo (DRC), where the authors suggested that the transmission of *S. fuelleborni* may have occurred when the hunted monkeys were eviscerated and the gut contents contaminated areas where humans lived [16]. Persistent occurrence of *S. fuelleborni* in human communities inhabiting the rural areas of Africa and dominance of this species in African and Asian NHP suggests possible ongoing cross-transmission; however, further research is required to support this with molecular evidence. Following coproscopy, eggs referred to as *S. fuelleborni* have been found in both humans and NHP living in the Dzanga-Sangha Protected Areas [57]. Possible transmission of *Strongyloides* spp. between humans and NHP in the same locality was investigated by analysing 18S rDNA and *cox1* sequences [20], though on a limited number of larvae. Despite *S. fuelleborni* being found in all studied hosts, haplotypes from great apes differed from those in humans. By contrast, *S. stercoralis* was detected only in humans. Two studies report occurrence of *S. fuelleborni* in researchers who returned from Africa, where they had contact with NHP [35,58]. In the first case, the researcher returned from Tanzania, where she worked with chimpanzees [35], while the second researcher—in DRC—studied bonobos (*Pan paniscus*) [58], which are also known hosts of *S. fuelleborni* based on eggs detected in the bonobo faeces [59].

In contrast to tropical Africa, *S. fuelleborni* is rarely reported in Asian human populations. Using partial 18S rDNA and ITS-1, *S. fuelleborni* was detected in a human working closely with wild orangutans in Borneo [49]. Independent studies confirmed identical genotypes of *S. fuelleborni* in NHP and rural Asian human populations. People in Thailand, who have frequent contact with wild long-tailed macaques (*Macaca fascicularis*) harboured *S. fuelleborni* 18S rDNA and *cox1* haplotypes, which were found later in wild long-tailed macaques sampled in Thailand and Laos [36,60]. Transmission was also suspected between captive pig-tailed macaques (*Macaca nemestrina*) and their owner, as they shared the same *S. fuelleborni* 18S rDNA and *cox1* haplotypes [37].

Similarly, transmission of *S. stercoralis* between captive NHP and zoo/sanctuary personnel and *vice versa* is very likely [21], facilitated by close contact in captive and semi-captive conditions [50]. *Strongyloides stercoralis* is commonly found in captive NHP in zoological gardens [50] and rehabilitation centres [49]. *Strongyloides stercoralis* infection was reported in wild great apes and humans sharing the same habitat in the Republic of the Congo [61]; however, this was concluded based only on a qPCR protocol using generic primers, which do not differentiate between *Strongyloides* species and therefore the results are highly speculative. Notably, humans as well as NHP are typically infected with *S. stercoralis cox1* lineage A, for which domestic dogs could possibly be a source of infection [17]. Thus, dogs could play a role in transmission of *S. stercoralis* to NHP as well, especially in areas of mutual habitat encroachment or captivity.

In the case of *S. cebus*, there is no known transmission between NHP and humans. A volunteer was experimentally infected with larvae of *S. cebus* from the capuchin monkey *Cebus capucinus hypoleucus*, but infection was not established in humans [30]. However, successful infections with *S. fuelleborni* were achieved after human exposure to larvae cultured from the faeces of various Old World NHP [30,62–64].

4. Pathogenicity of *Strongyloides* for non-human primates

Numerous studies describe fatal cases in captive animals [65–67] and it seems that these cases have been caused exclusively by *S. stercoralis* or *S. cebus*. In humans, most infections with *S. stercoralis* are mild, but the infection can lead to complicated strongyloidiasis under certain conditions (e.g.

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immunosuppression) [68]. While uncomplicated disease is manifested by gastrointestinal, pulmonary and dermatological symptoms, infection in immunosuppressed patients can lead to severe systemic disease known as disseminated strongyloidiasis, with possible fatal consequences [69]. NHP under the age of five years appear to be most susceptible to clinical disease [49]. A lethal course of *S. stercoralis* infection has also been noted in young chimpanzees and gorillas at the San Diego Zoo [65] when more than a quarter of a million filariform larvae were found at autopsy in one individual in all parts of its gastrointestinal tract, in the lungs and in the urinary bladder [65]. Disseminated strongyloidiasis caused by *S. stercoralis* was reported in five captive common patas monkeys (*Erythrocebus patas*) [70] and fatal strongyloidiasis (identified as *Strongyloides* sp.) in a 5-month-old captive Sumatran orangutan [71]. By contrast, no clinical signs were observed in captive orangutans infected by *S. stercoralis cox1* lineage A in Czech zoos [50]. When black-tufted monkeys (*Callithrix penicillata*) were subcutaneously inoculated with *S. stercoralis* of human origin [72], most animals showed no clinical signs but immunosuppressed individuals showed progressive disease leading to disseminated infection.

Clinical signs have also been reported in captive NHP with *S. cebus* infection. These included diarrhoea, weight loss, loss of appetite, dehydration and dyspnoea [40]. In fatal cases, post-mortem examination revealed inflammatory processes with a substantial presence of larvae and eggs in the pancreas, liver, heart and lungs, as well as lesions and haemorrhages in the small intestine and lungs [40].

Unlike *S. stercoralis* and *S. cebus*, asymptomatic infections with *S. fuelleborni* occur in free-ranging primates [21]. In experimental infections of humans with *S. fuelleborni* from NHPs, clinical symptoms often did not occur even though the infection was successful [30,62,63]. Infection with clinical symptoms has been described only in humans after exposure to *Strongyloides* larvae from gibbons [64]. Nevertheless, an apparently mild and self-limiting infection with *S. fuelleborni* has been described in local people in Central African Republic, who share an environment with NHP [73]. Clinical symptoms have also been observed in a person working with NHP in Tanzania [35]. In general, clinical signs, if present, are similar to those of *S. stercoralis* infection, such as localized skin symptoms, dry cough with possible tracheal irritation, abdominal pain and diarrhoea [73]. However, disseminated and fatal strongyloidiasis infections in humans have only been reported with *S. stercoralis*, which may be due to the capability of autoinfection, unknown in *S. fuelleborni*. While in *S. stercoralis* direct (homogony) and indirect (heterogony) cycles can alter, one indirect developmental cycle is necessary for *S. fuelleborni* [30].

5. Diagnostic methods for *Strongyloides* detection in primates

Despite fresh faecal smears being frequently mentioned in the literature (electronic supplementary material, table S1), the microscopy methods predominantly used for parasite detection in NHP are based on concentration techniques in various modifications [74]. Flotation methods commonly use sucrose, sodium nitrate and zinc sulfate solutions, while sedimentation techniques such as formalin–ethyl acetate and formalin–ether sedimentation are also employed. In contrast to diagnostics in humans, where sedimentation techniques and the Kato-Katz technique are routinely used for *Strongyloides* detection [75], flotation is the most commonly used method for parasite detection in veterinary medicine and, as such, predominates as a diagnostic tool for helminth detection also in captive NHP. Most quantification methods such as McMaster, FLOTAC and MiniFLOTAC© are also based on flotation. In many cases, faecal samples from NHP, especially those collected in the field, are preserved and examined later. Formalin-based preservation increases fragility of thin-walled nematode eggs that tend to rupture or collapse under high osmotic pressure in flotation solutions [57]. This is a well-documented limitation for detection of strongylid nematode eggs and applies also for the thin-walled *Strongyloides* eggs that can be consequently overlooked. Proper identification of the eggs of oviparous *Strongyloides* species represents another complication as they are commonly misidentified as the embryonated eggs of strongylids (i.e. hookworms), a factor that should be considered in any further analyses of published data. *Strongyloides stercoralis* larvae do not float easily, so they also cannot be detected using flotation-based techniques, stressing the need for sedimentation-based diagnostic techniques. In general, all microscopy-based methods have a very low detection rate for *Strongyloides* compared to the more specific methods [75] that are discussed below. Still, microscopy is the only non-molecular method that can easily distinguish *S. stercoralis* L1 larvae, from oval eggs containing U-shape larva in other *Strongyloides* species, including *S. fuelleborni*, *S. kellyi* and *S. cebus* in the examined faeces (figure 1). Unfortunately, neither larvoscopic nor larval culture methods are routinely used to detect parasites in NHP. Baermann larvoscopy or Koga agar plate culture are essential methods to detect the L1 larvae from fresh faeces [76], but as L1 develop within a few hours for all *Strongyloides* spp., this method does not provide a means for distinguishing species. Infective L3 larvae and free-living adults can be developed by culturing in sterile sawdust [77] or by using a Harada Mori filter paper culture, although distinguishing different species based on L3 larvae is impossible due to overlapping morphological characteristics. Taxonomy and species identification for *Strongyloides* are commonly based on the morphology of parasitic females, which are collected at the necropsy of hosts [40]. This is often impossible due to the protected status of the NHP or lack of facilities where necropsy could be safely performed. Nevertheless, despite molecular methods being prioritized, morphology-based approaches are crucial for clarifying taxonomic ambiguities and new species description. For example, in *S. fuelleborni*, two *cox1*-based lineages are formed—an African and an Asian strain [21]—suggesting that they may be two separate species, but morphological confirmation is essential for new species establishment.

Serological methods such as a range of ELISA tests are routinely used in humans [78]. Although these tests have relatively high sensitivity, specificity is less assured and cross-reactivity can occur in cases of coinfection with other parasites [79]. These tests are hard to apply in wild NHP, as it is not possible to obtain invasive samples such as blood, and to our knowledge serology has not been used for *Strongyloides* detection in captive NHP either.

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Figure 1. (*a*) *Strongyloides fuelleborni* egg from lowland gorilla faeces, Dzanga-Sangha Protected Areas, Central African Republic and (*b*) *S. stercoralis* L1 rhabditiform larva from captive Sumatran orangutan (*Pongo abelii*) that are both passed in the faeces.

A range of DNA-based diagnostic techniques that target 18S rRNA gene (18S rDNA; mainly hypervariable regions HVR-I and HVR-IV), 28S rRNA gene (28S rDNA), ITS-1 and *cox1* were introduced for *Strongyloides* diagnostics (electronic supplementary material, table S2). A qPCR protocol based on 18S rRNA gene was developed for detection and quantification of *Strongyloides* at the genus level [80]. Genus-specific 28S rDNA-based loop-mediated isothermal amplification (LAMP) allows detection of *Strongyloides* without temperature cycling and is therefore suitable for resource-limited conditions [81]. However, none of the above methods can be used for accurate species identification without subsequent sequencing. Considering the diversity of *Strongyloides* species occurring in primates and their epidemiology, host spectrum and host specificity, genetic characterization should be a standard for epidemiological studies as well as for case reports. The aforementioned genetic markers are suitable for discrimination to the species level [21]. However, recent studies discovered host preferences of different *S. stercoralis* lineages (possibly subspecies or even cryptic species) and geographical clusters of *S. fuelleborni* that were detected only by certain markers [21,82,83]. Specifically, lineages A and B of *S. stercoralis* were discriminated by *cox1*, and this separation is also reflected by HVR-IV of 18S rDNA, while HVR-I clustering is rather random and thus does not represent a suitable marker [83,84]. Suitability of the remaining two markers (28S rDNA and ITS-1) for characterization of the lineages is yet to be determined. In a study from Myanmar [22], 13 28S rDNA haplotypes from dogs and humans were identified and formed two clades—human/dog and dog only, corresponding to *cox1* clade I (now Lineage A) and clade II (Lineage B), respectively. However, there was a 28S haplotype H3 from the dog-only clade that was obtained from isolates that clustered in both *cox1* clades I and II. The topology of the 28S rDNA tree did not show clear distinction to two separate and strongly supported clades that would reflect the *cox1* topology. Therefore, this marker deserves further attention. Providing sequences of multiple loci, covering both mitochondrial and nuclear DNA, is crucial for building up public databases allowing for deep analyses [34].

Conventional PCR analyses followed by Sanger sequencing are suitable for the barcoding of isolated *Strongyloides* larvae [20,50], but molecular identification of *Strongyloides* spp. from total DNA extracted from faeces or cultures, which might contain multiple *Strongyloides* strains and/or species, requires more complex genotyping approaches. Next generation sequencing (NGS) is an effective tool for dealing with multiple infections [83]. A genotyping assay adapted for deep amplicon sequencing on the Illumina MiSeq platform targeting the mitochondrial *cox1* locus and 18S rDNA HVR-I and HVR-IV was designed and used for sequencing multiple *Strongyloides* haplotypes in faecal samples from different hosts, including humans [83] and NHP [46], but such methods await their use in epidemiological studies aimed at detecting transmission of *Strongyloides* species between humans and NHP. Portable third-generation sequencers such as MinION Oxford Nanopore enable genetic identification of nematodes from complex natural environments, including faecal samples [85], and represent a promising alternative for epidemiological studies of *Strongyloides* spp. The main limitations of such metagenomic approaches are the (un)availability of equipment and reagents in many endemic countries, necessity of thorough optimization of the protocols because the current protocol for the three loci is not perfect (63%, 80% and 68% of sequencing success rate for *cox1*, HVR-I and HVR-IV, respectively) and an incomplete reference library, as well as the required expertise for raw data processing.

6. *Strongyloides* distribution based on the literature and sequencing data

Initial estimates in 1989 indicated 100 million people to be infected with *Strongyloides* nematodes [86]; this increased to 370 million in 2013 [87] and was most recently (in 2020) estimated at 600 million people infected worldwide, focussed largely in tropical and subtropical regions [88] but with an ever more global distribution, excluding only the far north and south [89] (figure 2). This continuous increase in cases is more likely due to improvements in diagnostic methods than actual increases in infected persons [88]. Nevertheless, the number of people infected with *Strongyloides* tends to be underestimated due to the sustained use of inadequate methods with low detection rates. In the case of NHP, the

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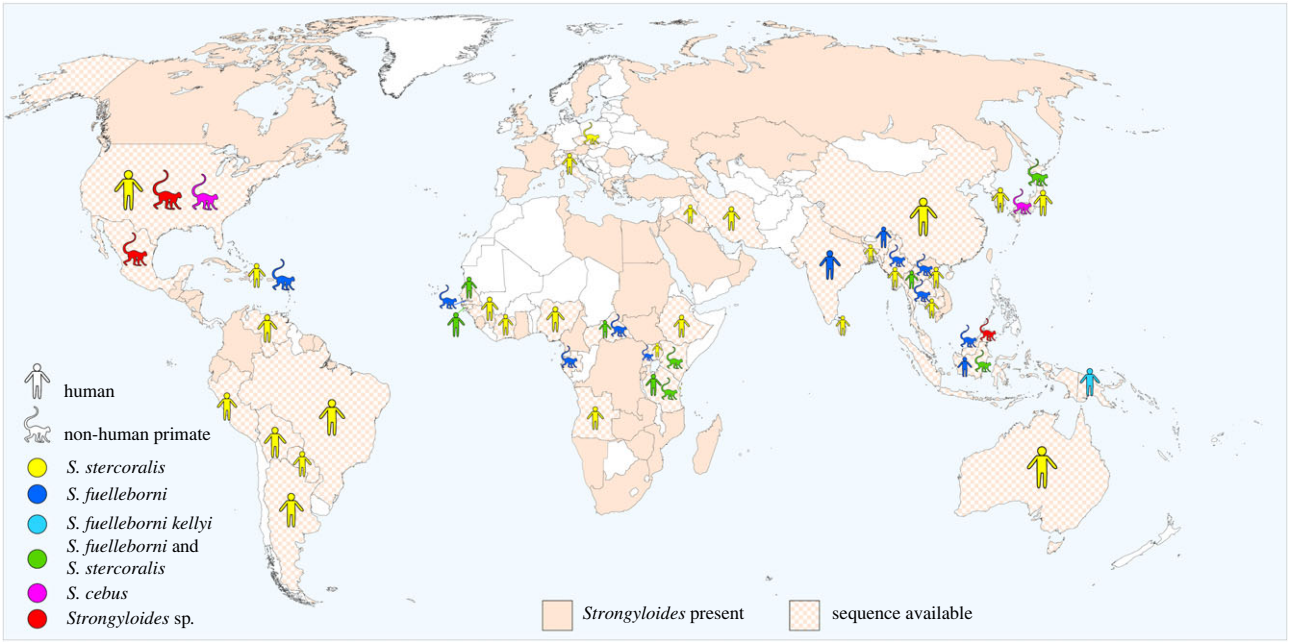


Figure 2. Geographical distribution of *Strongyloides* spp. in primates, including humans. Chequered filling marks the countries from which any DNA sequences are available in GenBank, together with the host's (human/non-human primate) and the parasite's identity. The overall distribution (presence of *Strongyloides* in a particular country) is based on several publications in humans [88–90], supplemented with data on the distribution of *Strongyloides* spp. in non-human primates.

global estimates of prevalence are lacking; available prevalence data range from <1% to 100% [91], being probably highly influenced by the methods used.

We searched all papers (search of the databases Web of Science and Scopus) dealing with NHP and parasites (keywords included parasites, *Strongyloides*, strongyloidiasis, primates and apes). Therefore, we not only entered the keyword *Strongyloides*, but also manually checked all papers on primate parasites, since many papers do not contain *Strongyloides* as a keyword and would be overlooked. All parasitological articles on NHP were manually checked and those reporting the presence of *Strongyloides* were included, so we finally selected and analysed 147 reports (electronic supplementary material, table A1). The majority of the studies present the overall parasite spectrum in investigated NHP and only a few publications addressed *Strongyloides* directly [20,44]. Most of the studies (73%) employed microscopy methods and *Strongyloides* was therefore classified as *Strongyloides* sp./spp. or to the assumed species based on presence of eggs or larvae. In Asia, mainly molecular-based identification data are available as all articles are recent, published in the past 15 years. Still, the number of publications is low in comparison with data from Africa, limiting information on the actual diversity and distribution of parasites in NHP, including *Strongyloides*, from this continent. In general, when *Strongyloides* is classified to the species level, *S. fuelleborni* is the most commonly cited species in African and Asian NHP (electronic supplementary material, table S1) [91], but it apparently occurs also in NHP introduced in the Americas [46]. In South America, data are primarily available from New World NHP from Brazil, Mexico and Peru. Coproscopic methods also dominate in these studies and therefore the *Strongyloides* species cannot be specified. Most of the data from African great apes, but also from other NHP, come from well-established research sites. A majority of the data are retrieved from the limited number of NHP populations that are habituated to frequent contact with humans. By contrast, a very limited amount of data is available from non-habituated animals.

The rise of molecular-based methods for *Strongyloides* detection is evident in both humans and NHP and their use has grown exponentially in the past few years [91]. This prompted us to investigate the *Strongyloides* spp. sequences obtained from humans and NHP that are available in the GenBank database (figure 2). Genomic DNA sequences labelled as *S. stercoralis*, *S. fuelleborni*, *S. cebus* and *Strongyloides* sp. and having a primate indicated as the host were downloaded from GenBank; commonly used markers such as 18S rDNA, ITS-1, 5.8S, ITS-2, 28S rDNA, *cox1*, 16S rDNA and complete mitochondrial genomes were included in the analysis ($n = 2347$). Sequences were imported to Geneious Prime 2023.1.2 (www.geneious.com) and sorted based on the marker, host species and country as provided in GenBank. In cases where the metadata were not provided, the publication linked to the sequences was searched for the relevant information.

The analysed *Strongyloides* sequences originated from 45 countries across Africa, Europe, Asia, the Americas and Australia (figure 2). The vast majority of sequences were labelled as *S. stercoralis* ($n = 1966$), mainly from humans ($n = 1662$) from 37 countries, with mostly *cox1* ($n = 1022$) sequences followed by 28S rDNA ($n = 696$) and 18S rDNA HVR-I ($n = 120$). The other hosts and markers were significantly less represented. Similarly, most of the *S. fuelleborni* ($n = 295$) sequences were *cox1* ($n = 203$). Almost all sequences were obtained from African and Asian NHP ($n = 272$), but *S. fuelleborni* was also detected in a colony of African vervet monkeys (*Chlorocebus aethiops sabaeus*) living on the Caribbean Island of St. Kitts [46]. As the Caribbean haplotypes clustered closely with the African isolates, it is likely that the parasites arrived with the animals originally brought to the island from Africa by Europeans in the seventeenth century [46]. The number of

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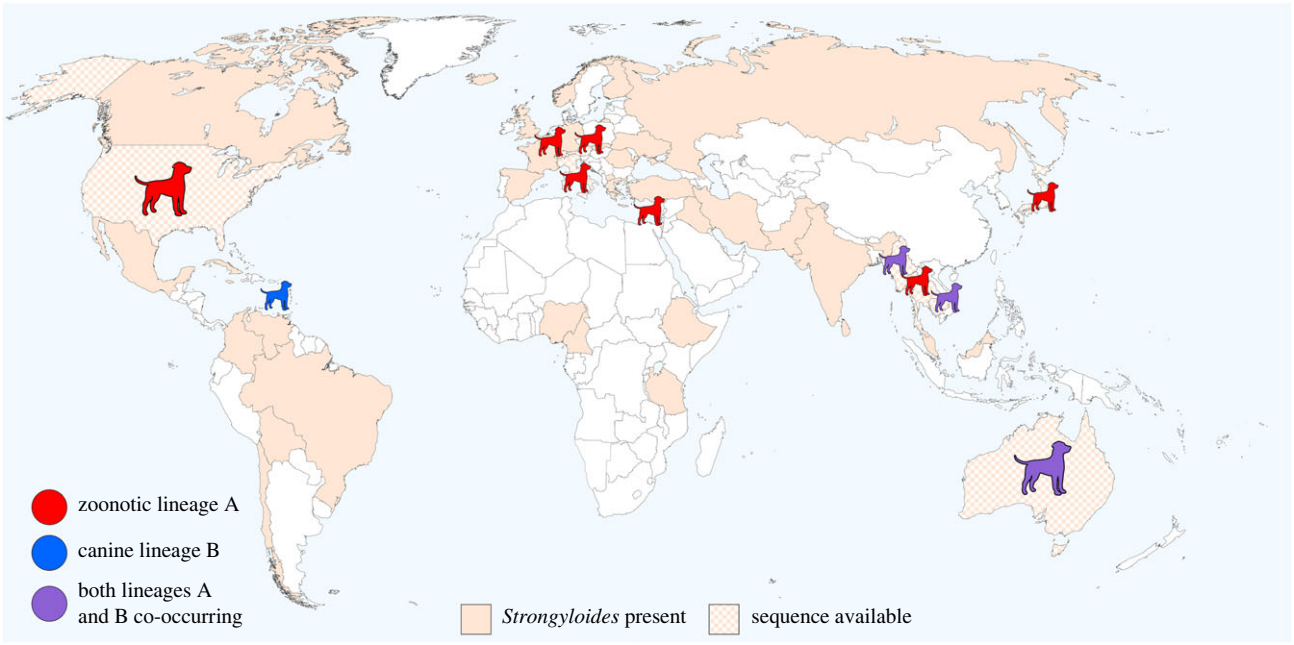


Figure 3. *Strongyloides stercoralis* in dogs. For chequered-filled countries, DNA sequences are available in GenBank. Lineages A and B are based on *cox1* and 18S rDNA HVR-IV as defined in Jaleta *et al*. [17]. The range of occurrence is based on several publications in dogs [91,92], supplemented with information from other studies about *Strongyloides* in dogs. There is an ongoing debate about whether the *Strongyloides* lineage B should be separated as a different species [68], considering *S. canis*, which was already proposed by Brumpt [93].

unidentified *Strongyloides* spp. sequences from NHP was significantly lower ($n = 325$) than from humans, but all three *Strongyloides* spp. known from NHP were represented in GenBank, with an additional three undetermined *Strongyloides* spp. from Bornean slow loris, spider monkeys and baboons. Twenty-three sequences of *S. fuelleborni* are available from humans from eight countries. Notably, only a single 18S rDNA HVR-I sequence is available for *S. kellyi*. There were also 46 sequences of *S. stercoralis* for which it was not possible to confirm the host species and/or locality.

Strongyloides sequences from Africa, but also from Europe, are clearly underrepresented in available datasets. Although the data from Asia and South America seem comparably more represented, they are mainly from a single study, a single sampling, or from multiple studies but focused on the same area (electronic supplementary material, table A2). Very little data are available from sites where humans and NHP share the same habitat. 18S rDNA and *cox1* regions are preferably targeted markers, however, the methods used were not uniform, making the phylogeny impossible or difficult to evaluate as different genetic markers are available for different species in GenBank (electronic supplementary material, table A2). Unfortunately, some studies have identified *Strongyloides* species/haplotypes using molecular methods, but have not entered sequences into databases [58], leaving them unavailable for further analyses.

Finally, considering that dogs could play a role in the dissemination of *Strongyloides*, we also investigated *Strongyloides* sequencing data available in GenBank from domestic dogs (figure 3) and their possible overlap with those from humans and NHP. Dogs frequently come into contact with NHP either as free-roaming intruders into natural habitats [94] or in villages and similar rural locations visited by NHP. *Strongyloides cox1* lineage A could likely be shared between dogs and humans [95] and therefore possibly also NHP. Analysing *Strongyloides* circulation among humans, dogs, and NHP is a critical gap in epidemiology, however, data enabling such analyses are not available. Notably, there are only seven countries (USA, Japan, Myanmar, Thailand, Cambodia, Japan, Australia and Italy) where sequences from dogs and humans (and also from NHP in the first four countries) are deposited in the GenBank, despite the fact that *Strongyloides* occurrence in canines and primates including humans, mostly overlaps (figures 2 and 3).

7. Message for *Strongyloides* control projects

Considering zoonotic features of strongyloidiasis, management of infection in the human population is a challenge and a research opportunity at the same time, deserving a One Health perspective. Our review has highlighted persisting knowledge gaps related to the diversity and global distribution of *Strongyloides* spp. in humans and NHP. To move forward in *Strongyloides* research, traditional microscopy-based techniques applied both in captive and free-ranging NHP should not be abandoned, but complemented with unified DNA-based tools shared among researchers on a global scale. The NGS metabarcoding (*cox1* and HVR-IV of 18S rDNA) appears as the pre-eminent scientific approach; however, there are significant obstacles to overcome such as success rate, demanding equipment and subsequent processing of the obtained data (see above). Elucidating the diversity of *Strongyloides* is critical for determining species and lineages that infect humans and for uncovering animal reservoirs, transmission pathways and impact on human health in areas of higher incidence of *Strongyloides* infections. Accurate diagnostics requires novel diagnostic assays (e.g. multiplex qPCR-based), that would allow the easy distinguishing *S. stercoralis*, *S. fuelleborni* and possibly *S. cebus* and *S. kelleyi* in the diagnostic routine. To date, we know little about the pathology of *S. fuelleborni* both in humans

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and NPH, or of *S. kelleyi* in humans. Considering that these 'non-stercoralis' taxa might dominate in some areas, such information is necessary for decision-making related to control of these infections.

In higher-incidence areas, active surveillance that involves not only samples from humans, but also from domestic dogs, sympatric NHP and eDNA is necessary to uncover sources of environmental contamination and to understand the role of animal reservoirs of human strongyloidiasis. Active penetration of *Strongyloides* larvae through the host's skin increases the transmission risk in shared environments. This applies not only for captive settings such as zoos, but also generally for a plethora of situations at the human–wildlife–domestic animals interface. Although experimental data confirming the transmission of *Strongyloides* between humans and NHP are very limited, the occurrence of identical haplotypes in humans and NHP indicates that the parasite can be shared among multiple hosts under natural conditions [20,37]. NHP frequently visit villages and feed on crops and could directly contaminate food or water sources. NHP are also traded and consumed as bushmeat or are kept as pets in villages and often played with by children. Importantly, terrestrial NHP were more likely positive for *Strongyloides* than the arboreal ones [96,97]; baboons as typically terrestrial primates are the most frequent visitors to human settlements in Africa [98]. In Asia, NHP play an important cultural role and are revered as deities, allowed to roam freely in villages and towns, visiting fields, parks, gardens and temples. Even people complying with hygiene standards and treated with anthelmintics can be infected/re-infected by *Strongyloides* shed by NHP in a shared environment. Preventing the NHP from entering the villages and human proximity is hardly achievable, but an effort should be made towards public awareness to minimize crop raiding, access of NHP to garbage and other food resources and intentional feeding of NHP by the public.

Possibility of repeated transmission from NHP and domestic dogs should be taken into account in all initiatives dealing with *Strongyloides* control in human populations. Regular dehelminthization is an advisable approach to control helminths in domestic dog populations [99]; however, there are no anthelmintic products formally licensed for treatment and prophylaxis of canine *Strongyloides* infections [100]. In the case of wildlife, such as NHP, administration of any anthelmintics has ethical constraints, including potential adverse impacts on the animals as well as on the environment [101], and the decision to medicate should not be made on the basis of individuals' own will or initiatives. Although human strongyloidiasis has recently been added to the list of neglected tropical diseases requiring control measures in endemic areas [102], effective treatment strategies are far from being optimized. Elimination of infection from an infected individual is difficult to achieve both in animals and humans; classical dosing of anthelmintics (e.g. ivermectin, albendazole and triabendazole) [103] is commonly not sufficient in the case of *Strongyloides*. In dogs, macrocyclic lactone-based anthelmintics show the desired effect [104] and the efficacy of ivermectin for the treatment of *S. fuelleborni* [105] and *S. cebus* [40] has also been demonstrated in NHP. Conversely, other data from captive NHP showed that treatment with anthelmintics does not effectively eliminate *Strongyloides* larvae shed in faeces [106]. The dosage and treatment regimen of ivermectin—the most appropriate drug to treat *Strongyloides*—need to be defined for both humans [103] and captive NHP [107], while the suitability of other macrocyclic lactones should be studied [108].

To conclude, we are still facing significant knowledge gaps concerning the diversity and distribution of *Strongyloides* spp., as well as its epidemiology and health consequences of resulting infections. We advocate for the adoption of a comprehensive and unified approach employing advanced molecular tools in the research and management of *Strongyloides* infection at the One Health platform, always considering zoonotic potential and possible animal reservoirs in the local contexts.

Data accessibility. The data are provided in electronic supplementary material [109].

Declaration of AI use. We have not used AI-assisted technologies in creating this article.

Authors' contributions. E.N.: data curation, formal analysis, writing—original draft, writing—review and editing; K.M.S.: data curation, validation, writing—review and editing; K.J.P.: data curation, funding acquisition, writing—review and editing; B.Č.: data curation, formal analysis, funding acquisition, writing—review and editing; D.M.: investigation, supervision, writing—review and editing; B.P.: conceptualization, data curation, writing—original draft, writing—review and editing.

All authors gave final approval for publication and agreed to be held accountable for the work performed therein.

Conflict of interest declaration. We declare we have no competing interests.

Funding. This work was supported by Czech Science Foundation grant no. 22-16475S, Morris Animal Foundation, grant no. D21ZO-042, U.S. Fish and Wildlife Service—Great Ape Conservation Fund, grant no. F22AP02231-00 and by institutional support from the Institute of Vertebrate Biology, Czech Academy of Sciences, Czech Republic (RVO:68081766) and Masaryk University (MUNI/A/1422/2022).

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Nosková, E., Modrý, D., Baláž, V., Červená, B., Jirků-Pomajbíková, K., Zechmeisterová, K., Leowski, C., Petrželková, K.J., Pšenková, I., Vodička, R., Kessler, S.E., Ngoubangoye, B., Setchell, J.M., Pafčo, B.

2023

Identification of potentially zoonotic parasites in captive orangutans and semi-captive mandrills: phylogeny and morphological comparison.

Am. J. Primatol. 85, e23475. https://doi.org/10.1002/ajp.23475

Check for updates

Received: 13 April 2022 | Revised: 12 December 2022 | Accepted: 23 January 2023

DOI: 10.1002/ajp.23475

RESEARCH ARTICLE

AMERICAN JOURNAL OF PRIMATOLOGY WILEY

Identification of potentially zoonotic parasites in captive orangutans and semi-captive mandrills: Phylogeny and morphological comparison

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Funding information
Masaryk University, Grant/Award Number: MUNI/A/1488/2021; Japan Society for the Promotion of Science-Czech Academy of Science bilateral cooperative program, Japan/Czech Republic, Grant/Award Number: JPJSBP120192506 and JSPS-19-17; Marie Sklodowska-Curie Individual Fellowship, Grant/Award Number: 703611; Institute of

Abstract

Cysts and trophozoites of vestibuliferid ciliates and larvae of *Strongyloides* were found in fecal samples from captive orangutans *Pongo pygmaeus* and *P. abelii* from Czech and Slovak zoological gardens. As comparative material, ciliates from semi-captive mandrills *Mandrillus sphinx* from Gabon were included in the study. Phylogenetic analysis of the detected vestibuliferid ciliates using ITS1-5.8s-rRNA-ITS2 and partial 18S ribosomal deoxyribonucleic acid (rDNA) revealed that the ciliates from orangutans are conspecific with *Balantioides coli* lineage A, while the ciliates from mandrills clustered with *Buxtonella*-like ciliates from other primates.

Abbreviations: BI, Bayesian inference; BLAST, Basic Local Alignment Search Tool; CIRMF, Centre Interdisciplinaire de Recherches Médicales de Franceville; GI, gastrointestinal; HVR, hypervariable regions; ITS, internal transcribed spacer; ML, maximum likelihood; PSD, pairwise sequence distance.

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Vertebrate Biology, Czech Academy of Sciences, Czech Republic,
Grant/Award Number: RVO:68081766

Morphological examination of the cysts and trophozoites using light microscopy did not reveal differences robust enough to identify the genera of the ciliates. Phylogenetic analysis of detected L1 larvae of *Strongyloides* using partial *cox1* revealed *Strongyloides stercoralis* clustering within the *cox1* lineage A infecting dogs, humans, and other primates. The sequences of 18S rDNA support these results. As both *B. coli* and *S. stercoralis* are zoonotic parasites and the conditions in captive and semi-captive settings may facilitate transmission to humans, prophylactic measures should reflect the findings.

KEYWORDS

Balantioides coli, *Buxtonella*-like, *Mandrillus sphinx*, molecular phylogeny, *Pongo abelii*, *Pongo pygmaeus*, semi-captive animals, *Strongyloides stercoralis*, zoo animals

1 | INTRODUCTION

Significant numbers of the gastrointestinal (GI) parasites reported in primates in both captive and free-ranging populations are potentially zoonotic (Islam et al., 2022; Labes et al., 2011; Sak et al., 2013). The close phylogenetic relationship between humans (*Homo sapiens*) and other primates further escalates the risk of potential pathogen transmission (including GI parasites). Such transmission occurs easily in conditions of close human-other primates contact, especially in captivity (Keita et al., 2014). In the wild, the risk of primate infection by pathogens originating from humans increases with increasing human pressure (Dunay et al., 2018; Kooriyama et al., 2013). Proximity to other animal species in captive settings may facilitate transmission of parasites with low host specificity and these animals may serve as reservoirs of infection for primates (Modrý & Escalante, 2018). Additionally, limited space in captive settings leading to close contact with animals creates opportunities for parasite transmission (Sanchez & Pich, 2018).

Vestibuliferid ciliates, traditionally referred as *Balantidium coli*, are highly prevalent GI parasites in captive orangutans (*Pongo pygmaeus*, *P. abelii*) (Nurcahyo et al., 2017). Until recently, the vestibuliferid ciliates (Ciliophora: Litostomatea: Vestibuliferida) of primates were all treated as a single species, *B. coli* (Malmstein, 1857), recently reclassified to the genus *Balantioides* (Chistyakova et al., 2014). However, Pomajbíková et al. (2013) confirmed the occurrence of another *Buxtonella*-like vestibuliferid ciliate species in primates. Using molecular tools, *Balantioides coli* has been detected in captive hamadryas baboons (*Papio hamadryas*), captive macaques (*Macaca fascicularis*, *M. mulatta*), captive African great apes (*Gorilla gorilla*, *Pan troglodytes*, *P. paniscus*) and free-ranging mountain gorillas (*Gorilla beringei beringei*) (Barbosa et al., 2017; Hassell et al., 2013; Pomajbíková et al., 2013). Other studies, including reports on orangutans revealed the presence of *B. coli* based on microscopic examination only (Kuze et al., 2010; Mul et al., 2007; Setchell et al., 2007; Supriadi et al., 2012) and the exact taxonomic determination may be incorrect. In contrast, *Buxtonella*-like ciliates have never been reported in apes using molecular analyses, but only in primates of the family Cercopithecidae (Pomajbíková et al., 2013; Yan et al., 2018). Molecular analyses of vestibuliferid ciliates traditionally relied on amplification of 18S ribosomal deoxyribonucleic acid (rDNA) and internal transcribed spacer (ITS) (Barbosa et al., 2017; Norman Grim et al., 2015; Pomajbíková et al., 2013; Ponce-Gordo et al., 2011). While the 18S rDNA gene reveals basic classification and relatedness of taxa on a higher level (Wright et al., 1997), the ITS regions is a highly polymorphic marker suitable for determining the species and evaluating intraspecific diversity (Pomajbíková et al., 2013; Ponce-Gordo et al., 2011).

Balantioides coli is classified as an intestinal commensal (Schuster & Ramirez-Avila, 2008), but can be pathogenic under certain conditions and cause clinical symptoms in humans and captive primates (Schovancová et al., 2013; Strait et al., 2012). In primates, infection may be asymptomatic or accompanied by dehydration, bloody or mucous diarrhea, weight loss, anorexia, lethargy, tenesmus, and rectal prolapse (Cockburn, 1948; Strait et al., 2012; Teare & Loomis, 1982). Some cases of balantidiosis can be even fatal (Lankester et al., 2008; Strait et al., 2012). The intensity of infection can be affected by a diet rich in starch (Schovancová et al., 2013) and also by stress, which generally leads to immunosuppression (de Oliveira et al., 2022). *Balantioides coli* is transmitted directly between individuals via the fecal–oral route, but one of the main sources of the infection can be domestic pigs (*Sus scrofa domestica*) and wild boars (*Sus scrofa*) (Schuster & Ramirez-Avila, 2008). Despite some knowledge about *B. coli* in primates, no information is available about whether infections of *Buxtonella*-like ciliates can manifest clinically in primates.

The genus *Strongyloides* (Nematoda: Chromadorea, Rhabditida) includes at least 50 species of parasitic rhabditid nematodes (Speare, 1989). *Strongyloides stercoralis*, *S. fuelleborni*, *S. cebus*, and several unidentified *Strongyloides* spp. are known to infect primates; however, the species greatly differ in their host spectrum (Bradbury et al., 2021). While *S. stercoralis* has a broad host specificity and has been detected in a range of hosts including humans, carnivores, and (mainly captive) primates, *S. fuelleborni* is restricted to primates (mostly free-ranging) with occasional spillover to humans

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(Bradbury et al., 2021). Studies using genotyping concluded that *S. stercoralis* likely originated in dogs (*Canis lupus familiaris*) and later adapted to human and other primate hosts (Barratt et al., 2019; Jaleta et al., 2017). The most commonly used markers for molecular analysis of *Strongyloides* include two hypervariable regions (HVR) of the 18S rDNA gene (HVR-I and HVR-IV) and a selected portion of the mitochondrial cytochrome c oxidase subunit 1 gene (*cox1*). These markers have been compared in different hosts and geographic regions, and haplotypes of the HVR-IV of 18S rDNA have been found to match *cox1* haplotypes (Bradbury et al., 2021). A few studies have used 28S rDNA and parts of ITS genes for *Strongyloides* detection (e.g., Labes et al., 2011; Solórzano-García & Pérez-Ponce de León, 2017), but the number of such sequences is rather small and therefore these markers are usually not used for further analyses.

Strongyloides infection can be asymptomatic, while uncomplicated disease is manifested by GI, pulmonary, and dermatological symptoms. In immunocompromised human patients, infection can result in a serious systemic disease with fatal consequences (Hasegawa & Pafčo, 2018; Nutman, 2017). There are no reports on the clinical outcomes of *Strongyloides* infections in free-ranging apes or other primate species, but reports describe fatal cases in captive animals (Hasegawa & Pafčo, 2018; Strait et al., 2012). Kleinschmidt et al. (2018) reported fatal strongyloidiasis in a 5-month-old Sumatran orangutan (*P. abelii*). A fatal course of infection has been also reported in young chimpanzees (*P. troglodytes*) and gorillas (*G. gorilla*) (Penner, 1981). Orangutans below the age of 5 years appear to be the most prone to clinical disease (Labes et al., 2011; McClure et al., 1973; Uemura et al., 1979).

Our study aimed to survey a spectrum of parasites found in captive Bornean orangutans (*P. pygmaeus*) and Sumatran orangutans (*P. abelii*) in Czech and Slovak zoological gardens. Special attention was paid to potentially zoonotic GI parasites, specifically *Strongyloides* spp. and vestibuliferid ciliates. We further determined if the orangutans were infected with zoonotic vestibuliferid ciliates *B. coli* or probably nonzoonotic *Buxtonella*-like ciliates and identified *Strongyloides* species/haplotypes. As comparative material for the vestibuliferid ciliates, we used fecal samples from semi-free ranging mandrills (*Mandrillus sphinx*) from Gabon. First, we predict that the dominant detected GI parasites in studied captive orangutans from Czech and Slovak zoological gardens are nematodes of the genus *Strongyloides* and vestibuliferid ciliates, since these parasites were most frequently found in captive orangutans (Nurcahyo et al., 2017). Second, we assumed that potentially zoonotic *B. coli* and *S. stercoralis* are present in captive orangutans studied, since these species have already been identified in other captive primates using molecular analyses (Nurcahyo et al., 2017; Pomajbíková et al., 2013). Last, if wild primates of the family Cercopithecidae harbor potentially nonzoonotic *Buxtonella*-like ciliates (Pomajbíková et al., 2013; Yan et al., 2018), we predict that these ciliates might be present in semi-free living mandrills. Detection and exact identification of parasites in captive settings is important, as unattended infections can cause serious health problems or even death in mostly young, old and weak individuals (Sanchez & Pich, 2018). Moreover, parasites with zoonotic potential may pose a risk to keepers and other staff working with captive animals. Detailed information about the parasites of captive animals is crucial to evaluate their impact on the health of free-ranging individuals, which is necessary for good conservation management.

2 | METHODS

2.1 | Animal ethics statement

Fecal samples from captive orangutans were obtained via a formal agreement with the zoo management, mandrill samples were obtained during routine health examinations. Noninvasive sample collection was accomplished following individual national regulations or guidelines for animal care. Each facility received the results of the parasitological examination. The research adhered to the American Society of Primatologists Principles for the Ethical Treatment of Nonhuman Primates.

2.2 | Sampling and coproscopic examination

Individual fresh fecal samples ($N = 14$) were collected from captive Bornean orangutans (*P. pygmaeus*) ($N = 7$), Sumatran orangutans (*P. abelii*) ($N = 6$), and one hybrid housed in Czech and Slovak zoological gardens by the zookeepers in March-July 2019. Animal health is monitored, and any clinical signs were recorded. Individual fecal samples ($N = 6$) from semi-free ranging mandrills (*M. sphinx*) housed at the Centre Interdisciplinaire de Recherches Médicales de Franceville (CIRMF) in Gabon were collected as a comparative material for analyses targeting the vestibuliferid ciliates in June–July 2017. A portion of each orangutan and mandrill fecal sample was divided and stored in two preservation media: 10% formalin (orangutan and mandrill samples), 96% ethanol (orangutan samples), and 90% ethanol (mandrill samples), while a third portion of the same orangutan sample was kept fresh for later analyses. All samples were then shipped to the laboratories of the Department of Pathology and Parasitology, University of Veterinary Sciences Brno, Czech Republic. Part of the fresh individual orangutan sample was examined by Baermann larvoscopy to collect *Strongyloides* larvae and another part of the same sample fixed in formalin was examined microscopically using modified Sheather's flotation and formalin-ether sedimentation method for detection and identification of GI parasites (Jirků-Pomajbíková & Hůzová, 2018; Pafčo, 2018). Cysts and trophozoites of vestibuliferid ciliates were measured under a light microscope (Olympus BX41) at 400 × magnification and photographed using Olympus AX70 with Nomarski differential contrast (Olympus, headquarters). Samples positive for vestibuliferid ciliates and *Strongyloides* sp. were selected for further DNA barcoding and phylogenetic analyses.

2.3 | DNA isolation, PCR, sequencing, and cloning

Fecal samples microscopically positive for vestibuliferid ciliates (orangutans $N = 4$, mandrills $N = 6$) preserved in ethanol were dried

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overnight at 37°C before DNA isolation to evaporate the ethanol. The total DNA was extracted using DNeasy PowerSoil Kit (QIAGEN) following the manufacturer's protocol. The partial 18S rDNA gene (1270 bp) of the ciliates was amplified using EukA forward and SSUrBB reverse primers (Norman Grim et al., 2015) using Emerald AMP polymerase (Takara), while the ITS1-5.8s-rRNA-ITS2 region (ITS; 400 bp) was amplified using B5D forward and B5RC reverse primers (Ponce-Gordo et al., 2011) using Top Bio PPP polymerase (Top-Bio, s.r.o.). The total volume of 25 µL PCR mixture for 18S rDNA contained 12.5 µL polymerase, 1.25 µL of 10 µM each primer. The total volume of PCR mixture for ITS was the same and contained 12.5 µL polymerase and 1 µL of 10 µM each primer. Template DNA volume was 2 µL.

Individual *Strongyloides* larvae ($N = 15$) were collected from orangutan fecal samples ($N = 4$) after the Baermann larvoscopy method (Pafčo, 2018), placed in microcentrifuge tubes and frozen. Invisorb® Spin Forensic Kit (STRATEC) was used to isolate DNA from individual *Strongyloides* larvae following the manufacturer's protocol. Three genetic markers were selected for amplification: *cox1* and two HVR of 18S rDNA (HVR-I and HVR-IV) according to Barratt et al. (2019). Two primer pairs: TJ5207 forward and TJ5208 reverse (Jaleta et al., 2017) and SPPcox1F forward and SPPcox1R reverse (Barratt et al., 2019) were used for amplification of 650 and 217 bp of *cox1*, respectively. The total volume of 25 µL PCR mixture for *cox1* contained 12.5 µL Top Bio PPP polymerase (Top-Bio, s.r.o.), 0.5 µL of 10 µM each primer (TJ5207, TJ5208) or 12.5 µL PrimeStar polymerase (Takara) with 1.25 µL of 10 µM each primer (SPPcox1F, SPPcox1R). Template DNA volume was 2 µL. Two 18S rDNA HVR were amplified using primer pairs New HVRI F forward and New HVRI R reverse (HVR-I, 434 bp) and New HVR-IV F forward and New HVRIV R reverse (HVR- IV, 255 bp) (Barratt et al., 2019) using PrimeStar polymerase (Takara). The total volume of 25 µL PCR mixture for HVR-I contained 12.5 µL PrimeStar polymerase (Takara), 1.5 µL of 10 µM each primer. For HVR-IV, the total volume of PCR mixture was the same and contained 12.5 µL PrimeStar polymerase (Takara) and 0.5 µL of 10 µM each primer. Template DNA volume was 2 µL.

Amplification of all targeted *Strongyloides* and vestibuliferid ciliates markers was performed using Biometra T-personal thermocycler (Schoeller) under conditions specified in Table 1. The PCR products were separated by electrophoresis in a 1.5% agarose gel with Midori Green Advance (Nippon Genetics) and visualized on a UV transilluminator. The PCR products of the expected size were purified either directly or after cutting the band from the gel using Gel/PCR DNA Fragments Extraction Kit (Geneaid Biotech). All amplicons were Sanger sequenced in both directions commercially by Macrogen Europe (Amsterdam, Netherlands). If we detected mixed signals, the PCR product was cloned using pGEM® -T Easy Vector Systems (Promega Corporation) and GenElute™ Plasmid Miniprep Kit (Sigma-Aldrich). The resulting cloning products were also sent for Sanger sequencing. All obtained sequences were checked and manually trimmed in Geneious 9.1.5 (www.geneious.com) and the identity was checked using BLAST (Altschul et al., 1990).

2.4 | Phylogenetic analyses

All sequences obtained were aligned with sequences from GenBank using Muscle alignment in Geneious 9.1.5 (Kearse et al., 2012). For the 18S rDNA of vestibuliferid ciliates, the alignment (1629 bp) consisted of seven sequences obtained during the present project and

TABLE 1 PCR primers and reaction conditions.

Primers	Sequence 5′→ 3′	Reaction conditions	Region of gene	Parasite	References
EukA	AACCTGGTTGATCCTGCCAGT,	10 min at 94°C; 35 cycles of 1 min at 94°C, 90 s at 54.9°C, 90 s at 72°C; and 5 min at 72°C.	18S rDNA	Vestibuliferid ciliates	Norman Grim et al. (2015)
SSUrBB	AAATACATAGTCCCTCTAAGAAGTC				
B5D	GCTCCTACCGATACCGGGT,	10 min at 94°C; 30 cycles of 1 min at 94°C, 1 min at 64°C, 1 min at 72°C; and 5 min at 72°C.	ITS1-5.8s-rRNA-ITS2	Vestibuliferid ciliates	Ponce-Gordo et al. (2011)
B5RC	GCGGGTCATCTTACTTGATTTC				
TJ5207	TTTGATTGTTACCTGCTTCTATTTT	2 min at 94°C; 35 cycles of 20 s at 94°C, 15 s at 50°C, 90 s at 72°C; and 7 min at 72°C	*cox1*	*Strongyloides* sp.	Jaleta et al. (2017)
TJ5208	TTTTACACCAGTAGGAACAGCAA				
SPPcox1F	TTTGATCCTAGTTCTGGTGGTAATCC	2 min at 98°C; 45 cycles of 10 s at 98°C, 10 s at 63°C, 40 s at 72°C; and 4 min at 72°C	*cox1*	*Strongyloides* sp.	Barratt et al. (2019)
SPPcox1R	GTAGCAGCAGTAAAATAAGCACGAGA				
HVRI F	GCTCATTATAACAGCTATAGACTACACGGTA		18S rDNA (HVR-I)	*Strongyloides* sp.	Barratt et al. (2019)
HVRI R	CCACAACAATCATTTTATGCACTTGG				
HVRIV F	CGGGCCGGACACTATAAGG		18S rDNA (HVR-IV)	*Strongyloides* sp.	Barratt et al. (2019)
HVRIV R	ATCTCTAAACAGGAACATAATGATCACTAC				

Abbreviations: HVR, hypervariable region; ITS, internal transcribed spacer; PCR, polymerase chain reaction; 18S rDNA, ribosomal deoxyribonucleic acid.

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82 sequences from Genbank. *Epispathidium papilliferum* (DQ411857) was used as an out-group. The final alignment of vestibuliferid ciliates ITS (534 bp) region sequences consisted of 11 newly obtained sequences and 94 sequences from the Genbank database representing genera *Balantioides*, *Buxtonella*, *Buxtonella*-like and *Balantidium* with *Balantidium ctenopharyngodoni* (KU170972) used as an out-group. Nodal support was assessed to 10^6 replicates. The phylogenetic relationships were reconstructed by Bayesian inference (BI) in MrBayes 3.2.6 (Huelsenbeck & Ronquist, 2001) with the substitution model GTR + G.

Four *Strongyloides cox1* sequences newly obtained from orangutan fecal samples were aligned with 56 homologue sequences representing the genus *Strongyloides* from GenBank with the length of the final alignment being 722 bp. *Necator americanus* (AJ417719) was used as an out-group. Nodal support was assessed to 10^6 replicates. Phylogenetic relationships were reconstructed by BI and Maximum likelihood (ML) approaches in MrBayes 3.2.6 (Huelsenbeck & Ronquist, 2001) and PhyML 3.3.2 (Guindon et al., 2010) programs. Substitution model GTR + G was used as it was selected by Modelgenerator (Keane et al., 2006).

3 | RESULTS

3.1 | Potentially zoonotic parasites detected in captive orangutans

In total, 14 fecal samples of individual orangutans from five Czech and Slovak zoos were microscopically examined. Four samples were negative for any parasites/commensals (Table 2). Cysts and trophozoites of vestibuliferid ciliates, tentatively identified as *B. coli* based on morphology, were found in four orangutan samples. Further, cysts of *Entamoeba coli*, *Chilomastix* sp., and *Giardia* sp. were detected (Table 2). *Entamoeba coli* was the most prevalent parasite, with cysts found in seven samples (50%), although in low quantities. Cysts of *Chilomastix* sp. and *Giardia* sp. were detected in a single individual each (Table 2). Baermann larvoscopy revealed *S. stercoralis* L1 larvae in four orangutan fecal samples. Both *B. coli* and *S. stercoralis* were detected in three samples, while two orangutans were each infected with *S. stercoralis* or *B. coli* only.

3.2 | No clinical signs related to parasitic infections were observed

None of the individuals positive for vestibuliferid ciliates and/or *Strongyloides* and no animals in the group showed any clinical signs at the time of sampling, or long term.

3.3 | Morphology of the vestibuliferid ciliates

Cysts and trophozoites corresponding morphologically to *B. coli* were found in four orangutan samples. Three samples contained only cysts, while both cysts and trophozoites were found in one sample, though trophozoites were in the minority (approx. 10%). The cysts were spherical or slightly subspherical with clearly visible vacuole

TABLE 2 Gastrointestinal parasites detected by microscopical observation of fecal samples of captive orangutans from Czech (CZ) and Slovak (SK) zoos.

Country/location	Sex	Host species/sample ID	*Strongyloides stercoralis*	*Balantioides coli*	*Entamoeba coli*	*Chilomastix* sp.	*Giardia* sp.
CZ/DKnL	F	*Pongo pygmaeus*/1	+	+	+	–	–
	F	*P. pygmaeus*/2	+	+	–	–	–
CZ/UnL	F	*P. pygmaeus*/3	–	–	+	–	–
	F	*P. pygmaeus*/4	–	–	–	–	–
	M	Hybrid/5	–	–	–	–	–
SK/Bojnice	M	*P. pygmaeus*/6	–	–	+	–	–
	F	*P. pygmaeus*/7	–	–	+	–	–
	M	*P. pygmaeus*/8	–	–	–	–	–
CZ/Praha	F	*P. abelii*/9	–	–	+	+	–
	F	*P. abelii*/10	+	–	+	–	+
	M	*P. abelii*/11	–	–	+	–	–
SK/Bratislava	M	*P. abelii*/12	+	+	–	–	–
	F	*P. abelii*/13	–	+	–	–	–
	M	*P. abelii*/14	–	–	–	–	–
Total prevalence			28.6% (4/14)	28.6% (4/14)	50% (7/14)	7.1% (1/14)	7.1% (1/14)

Abbreviations: DKnL, Dvůr Králové nad Labem; UnL, Ústí nad Labem; +, positive sample; –, negative sample.

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containing ingested starch (Figure 1a). In total, 98 cysts were measured with a mean length of 50 µm (range 34.2–68.0) and width of 49 µm (range 35.1–70.9). The trophozoites were ovoid with visible somatic cilia and apparent cytostome (Figure 1b). Five intact trophozoites were measured with a mean length of 52 µm (range 45.0–57.5) and a mean width of 45 µm (range 36.8–63.7).

Vestibuliferid ciliates were found in all six mandrill samples examined. Either cysts or trophozoites, or mixture of both were observed. Examined cysts ($N = 98$) were spherical or slightly subspherical and measured a mean of 44 µm (range 26.3–69.4) × 42 µm (range 24.8–66.9). The cysts had a thick transparent wall tightly encompassing the visible trophozoite inside. The somatic ridges on the surface of the encysted trophozoites ran in parallel rows, resembling a fingerprint (Figure 1c). A vacuolar structure was also present centrally or sub-centrally in the majority of the observed cysts. Trophozoites were ovoid, covered by rows of somatic cilia with cytostome localized at the anterior end of the body and contained food vacuoles (Figure 1d). The trophozoites ($N = 33$) measured a mean of 68 µm (range 38.7–105.9) × 40 µm (range 15.6–71.5). When visible, their cytostome measured a mean of 17 µm (range 12.1–19.7) long.

3.4 | Phylogenetic analysis of vestibuliferid ciliates

Seven sequences (1145–1170 bp) of partial vestibuliferid ciliates 18S rDNA were obtained (two from Bornean orangutans, one from Sumatran orangutan, and four from mandrill samples). All sequences from orangutans were identical. In mandrills, one sequence differed by 2.8%. Sequences from orangutans differed from mandrill-derived sequences by 3.3–4.2%.

The resulting BI tree showed 19 separate clades corresponding to individual genera (Figure 2). All the isolates thought to be *B. coli* from orangutans clustered within a highly supported (1.00 BI) sub-clade within the genus *Balantioides* clade comprising mainly isolates from other captive primates: western lowland gorillas (*Gorilla gorilla gorilla*), chimpanzees (*Pan troglodytes*), a bonobo (*P. paniscus*), and a hamadryas baboon, a few sequences from pigs (*Sus scrofa domestica*), from an ostrich (*Struthio camelus*), a common rhea (*Rhea americana*), and a Guinea pig (*Cavia porcellus*) (Figure 2). The other *Balantioides* sub-clade comprised sequences mainly from pigs, an Asian elephant (*Elephas maximus indicus*), a giant anteater (*Myrmecophaga tridactyla*), and a chimpanzee. The pairwise sequence distance (PSD) within the *Balantioides* clade did not reach over 2.1%.

In contrast, the sequences originating from mandrills clustered within a highly supported (0.9 BI) clade comprising only sequences labeled as *Buxtonella*-like and originating from various cercopithecids. One sequence from a mandrill (OM423619) clustered separately from all other isolates in a highly supported (1.00 BI) sub-clade (Figure 2). The sequence differed from the sequences within the major sub-clade by 2.7–3.2%, while the PSD within the major sub-clade was below 0.3%.

Eleven sequences (420–514 bp) of ITS were obtained during the current study (two sequences from Bornean orangutans, five clones of an isolate from a Sumatran orangutan, and four sequences from mandrill samples). All sequences from Bornean orangutans were 100% identical. In the Sumatran orangutan, the cloned sequences differed by 0.2–1.6%. In mandrills, one sequence differed by 6%. Sequences from orangutans differed from mandrill-derived sequences by 8.4–11%. The ITS BI tree topology was similar to that of the 18S rDNA but showed significant intraspecific variability, mainly in the genera *Balantioides* and *Buxtonella*-like. The resulting tree showed four separated clades corresponding to the genera *Balantioides*, *Buxtonella*-like, *Buxtonella*, and *Balantidium* (Figure 2). All isolates from orangutans clustered within a highly supported sub-clade (1.00 BI) corresponding to the sub-clade A *sensu* Ponce-Gordo et al. (2011). This sub-clade A comprises sequences obtained from captive primates, one free-ranging mountain gorilla (HF545448), pigs,

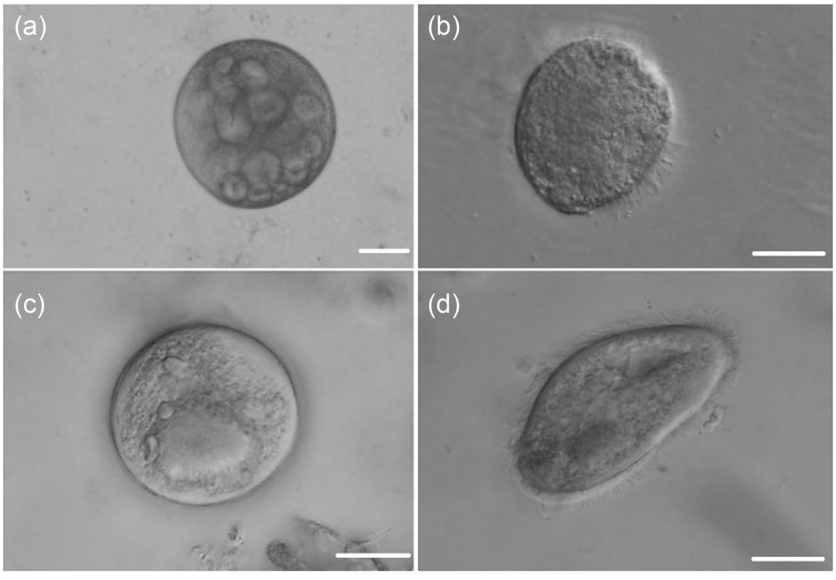


FIGURE 1 Life stages of vestibuliferid ciliates found in formalin-preserved fecal samples from captive orangutans from Czech and Slovak zoos and semi-captive mandrills from Gabon; scale bar = 20 µm. (a) *Balantioides coli* cyst from a Bornean orangutan with clearly visible granule of ingested starch; (b) *B. coli* trophozoite, Bornean orangutan; (c) *Buxtonella*-like cyst from a mandrill with a visible large "vacuole" inside; (d) Trophozoite of *Buxtonella*-like ciliate, mandrill.

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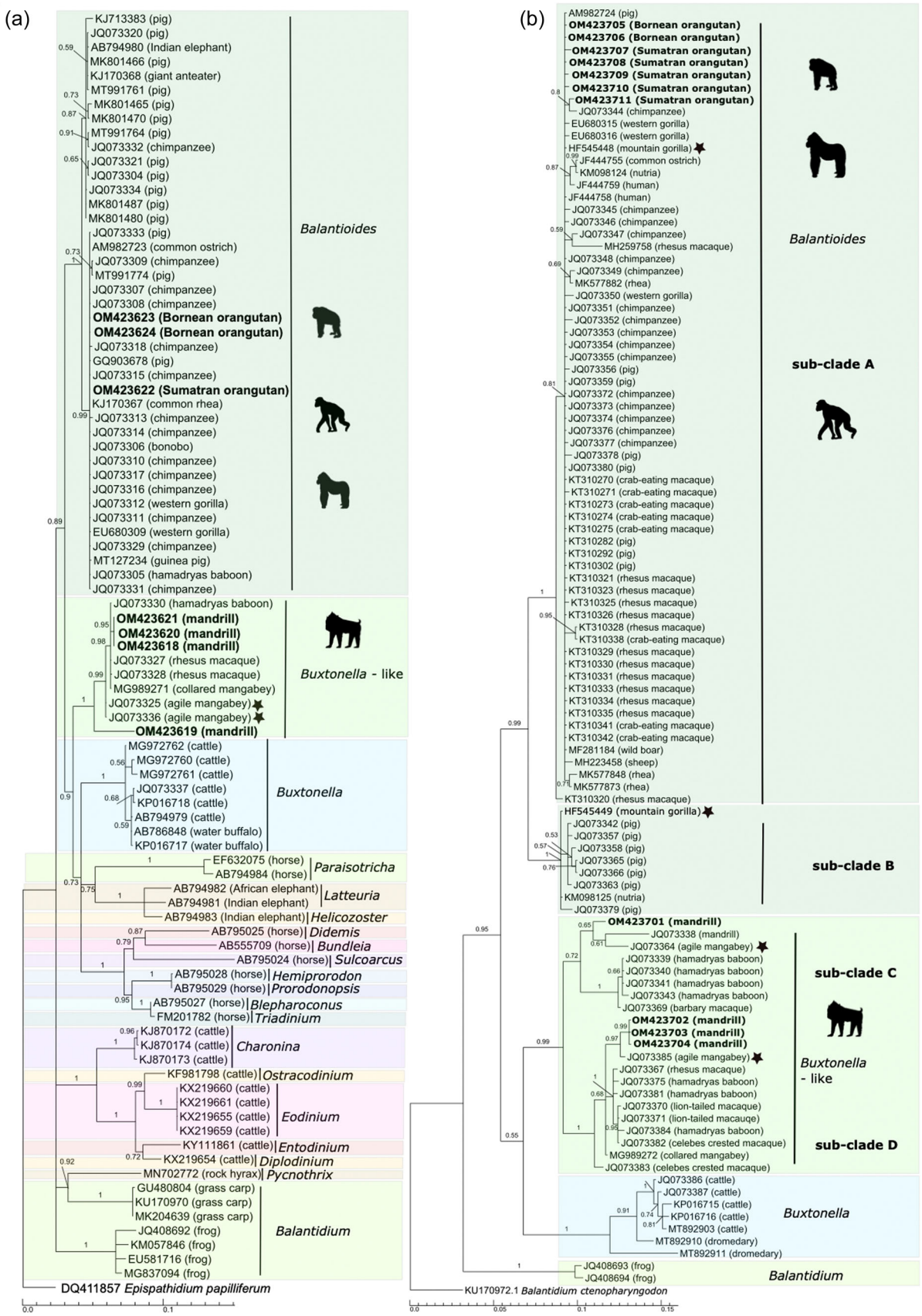


FIGURE 2 Bayesian inference phylogenetic tree inferred from (a) 18S rDNA (1629 bp) and (b) ITS region (534 bp). Both trees were calculated from a muscle-constructed alignment using GTR + G model for nucleotide substitutions. Numbers at the branches indicate Bayesian posterior probability based on 10^6 replicates. Sequences obtained during the current study are in bold. Branch lengths indicate expected numbers of substitutions per nucleotide site. The asterisk indicates free-ranging hosts.

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wild boars (*Sus scrofa*), paleognath birds, humans, sheep (*Ovis aries*), and nutria (*Myocastor coypus*). Sub-clade B *sensu* Ponce-Gordo et al. (2011) was further divided by a polytomy and comprised mostly closely clustering sequences from domestic pigs and then rather distant sequences from a nutria, free-ranging mountain gorilla (HF545449) and one more pig sequence. The clades *Balantioides* and *Buxtonella*-like differed by 2.4–5.2%, while the PSD observed among other genera varied between 2.9% and 11.3%.

The four ciliate sequences originating from mandrills clustered with other ciliates from cercopithecids within a highly supported (0.99 BI) clade of *Buxtonella*-like ciliates, which was further divided into two further structured sub-clades which we labeled C and D (Figure 2). Sub-clade C included closely clustering sequences from captive hamadryas baboons and barbary macaque (*Macaca sylvanus*) and rather distant cluster of one of our mandrill-derived sequences (OM423701, originating from the same sample as the different 18S rDNA sequence—OM423619), another sequence from a captive mandrill and a free-ranging agile mangabey (*Cercocebus agilis*) from the Central African Republic. The sequences within sub-clade C differed by 0.4–6.3%. Sub-clade D comprised sequences from captive baboons, captive macaques, captive collared mangabey, and the remaining sequences from our mandrill samples which clustered closely with a sequence from wild agile mangabey (Figure 2). PSD in this sub-clade did not exceed 11.4%, while sub-clades C and D differed by 5–11.1%. All sequences of vestibuliferid ciliates obtained in this study were uploaded to GenBank under the accession numbers OM423618–OM423624 (18S rDNA); OM423701–OM423711 (ITS1-5.8s-rRNA-ITS2) (see Supporting Information: Table S1 for details).

3.5 | Haplotypes and phylogenetic analysis of *S. stercoralis*

Four sequences of partial *cox1* (263–627 bp) and four sequences of each 18S rDNA HVR-I (391–457 bp) and HVR-IV (235–281 bp) were obtained from individual *S. stercoralis* larvae extracted from orangutan fecal samples (two from *P. pygmaeus* and two from *P. abelii*). A BLAST search showed that all four sequences of HVR-IV corresponded to haplotype A in Barratt et al. (2019), while in HVR-I region, haplotype VI was detected in *P. pygmaeus* and haplotype II in *P. abelii*, following Barratt et al. (2019). The two *S. stercoralis cox1* sequences from *P. abelii* were identical, while the two sequences from *P. pygmaeus* differed at a single-nucleotide polymorphis site. The PSD between the *cox1* sequences from the two orangutan species was 2%. Both BI and ML *cox1* phylogenetic trees were well-resolved with highly supported nodes and yielded the same general topology (Figure 3). Clades formed corresponding to individual *Strongyloides* species. All *S. stercoralis* sequences obtained from orangutan fecal samples clustered in the highly supported (1.00 BI) clade corresponding to lineage A *sensu* Jaleta et al. (2017) (highlighted in darker blue in Figure 3) infecting dogs, humans, and other primates. The clade of lineage A was further divided into several sub-clades. The sequences from Bornean orangutans were placed in a different sub-clade than sequences from Sumatran orangutans and these two sub-clades differed by 1.7–3.6%. The PSD within the sub-clades comprising the Bornean orangutan sequences and Sumatran orangutan sequences was below 2.9% and 3.1%, respectively. All sequences of *Strongyloides* obtained in this study were uploaded to GenBank under the accession numbers OM423625–OM423632 (18S rDNA); OM392049–OM392052 (*cox1*) (see Supporting Information: Table S1 for details).

4 | DISCUSSION

Regular monitoring of parasites in captive primates including their molecular identification and genotyping should be an important part of health management, which can lead to reduction of the risk of parasite transmission between humans and other primates. However, genetic analyses are not routinely performed, and surveys focused on parasite infection in captive primates are rarely published and shared with a broad scientific audience. Here, we present a study focused on selected GI parasites with zoonotic potential in orangutans housed in Czech and Slovak zoos and provide important DNA analysis-based data for future research.

Parasites of orangutans are overall much less studied than those of African great apes (Modrý & Escalante, 2018). Captive, semi-captive, and wild orangutans are commonly diagnosed with protists and helminths with relatively low host specificity often in combination with orangutan-specific parasites (Modrý & Escalante, 2018). We detected *Giardia intestinalis*, which is a typical parasite with low host specificity. Infections in primates can occur asymptomatically or with clinical symptoms such as diarrhea or vomiting (Strait et al., 2012) that were not observed during our study. We also detected *E. coli* and *Chilomastix* sp., which are also protists commonly occurring in great apes (Jirků-Pomajbíková & Vlčková, 2018; Kváč & McEvoy, 2018). Both protists occur naturally in primates and are not pathogenic to the host (Jirků-Pomajbíková et al., 2016; Kváč & McEvoy, 2018). However, the most commonly detected GI parasites in captive and free-ranging orangutans are nematodes of the genus *Strongyloides* and vestibuliferid ciliates *B. coli* (Foitová et al., 2009; Nurcahyo et al., 2017), both of which were among the most common parasites in our study, consistent with our first hypothesis.

The common occurrence of ciliates identified as *B. coli* is reported in most of the microscopy-based studies focused on parasitofauna of orangutans (e.g., Kilbourn et al., 2003; Kuze et al., 2010; Labes et al., 2011; Mul et al., 2007). Studies of mandrills, the source of our comparative material, also mention detection of *B. coli* (Poirotte et al., 2016; Setchell et al., 2007). However, recent studies (Chistyakova et al., 2014; Jirků-Pomajbíková & Modrý, 2018; Pomajbíková et al., 2013; Yan et al., 2018) and our results show that at least two taxa of vestibuliferid ciliates, specifically *B. coli* and *Buxtonella*-like ciliates, occur in primates. Due to the lack of clear identification structures on the ciliate's cysts, the two taxa can hardly be distinguished by their morphology. Thus, some of the published

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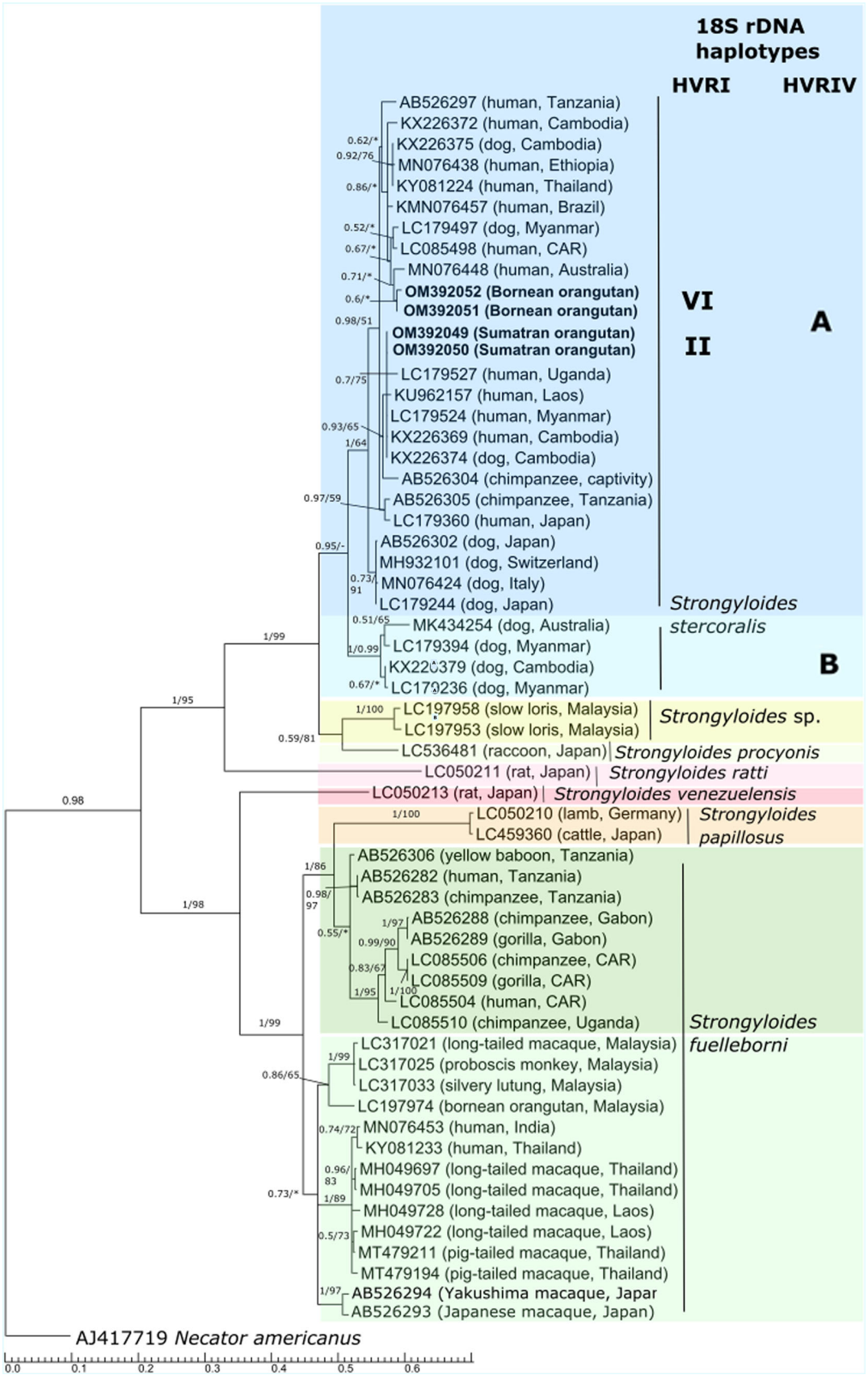


FIGURE 3 (See caption on next page)

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records of *B. coli* in primates may represent the latter group of vestibuliferids (*Buxtonella*-like ciliates).

Our phylogenies of the 18S rDNA and ITS regions showed almost identical results and clearly assigned the vestibuliferid ciliates of orangutans in the sub-clade A of *B. coli*, consistent with our second hypothesis. This was previously detected in various primates, including captive African great apes, several captive macaque species (Barbosa et al., 2017; Pomajbíková et al., 2013), and one isolate from a free-ranging mountain gorilla (Hassell et al., 2013), extending the spectrum of this sub-clade hosts to captive Bornean and Sumatran orangutans. In contrast, the ciliates from our comparative samples from mandrills clustered with *Buxtonella*-like ciliates from other cercopithecids, consistent with our last hypotheses.

Although many studies report the occurrence of *B. coli* in captive/semi-captive and free-ranging orangutans (Nurcahyo et al., 2017), supporting molecular data is lacking. Here, we, for the first time, performed molecular-phylogenetic analyses and confirmed the occurrence of *B. coli* in captive Bornean and Sumatran orangutans. In the past, it was impossible to distinguish whether orangutans (including captive, semi-captive, and wild) are infected by *Buxtonella*-like or *B. coli*. *Balantoides coli* is often detected in captive primates, including great apes (Pomajbíková et al., 2010). In contrast, *Buxtonella*-like ciliates seem to be restricted to primates of the family Cercopithecidae both in captivity and in the wild (Pomajbíková et al., 2013; Yan et al., 2018), which is supported by our results for semi-captive mandrills. So far, it appears that *B. coli* is less host-specific and may occur in various primates as well as in humans, while *Buxtonella*-like infects mostly primates other than great apes. Implementation of molecular phylogeny is necessary to clarify the distribution and host specificity of the two vestibuliferid ciliates in wild, semi-captive and captive primate populations.

The occurrence of potentially pathogenic and zoonotic *B. coli* in captive primates in zoos and occasionally in sanctuaries is noteworthy and deserves further attention. The parasite appears to be rarely detected in free-ranging great apes (Hassell et al., 2013), whereas in captivity, there may be factors that increase the susceptibility of primates to infection. In case of *B. coli*, there is a positive correlation recorded between the number of ciliates in fecal samples of captive chimpanzees and starch content in the diet (Schovancová et al., 2013). Optimal nutrition of great apes in (semi)-captive settings is of utmost importance to prevent metabolic diseases, dental problems, or abnormal behavior patterns (Edwards & Ullrey, 1999; Kuhar et al., 2013; Plowman, 2013). Therefore, the role of diet in orangutans infected with *B. coli* should be investigated in future studies.

Sequencing confirmed the existence of the two types of ciliates in our sample set. However, the question arises as to the identification of the two taxa based on their morphological characteristics observed under the microscope. In general, the cysts formed by vestibuliferid ciliates vary in size and shape even within a species, which complicates their identification (Kuze et al., 2010; Mul et al., 2007; Pafčo et al., 2018; Yan et al., 2018). Although the cysts appeared slightly different in microscopy (see large vacuole in the cysts of *Buxtonella*-like vestibuliferids, Figure 1c), we did not find robust features that distinguish between *B. coli* and *Buxtonella*-like ciliates in primate fecal samples using microscopic analyses.

From the clinical perspective, strongyloidiasis is the most important parasitosis of captive and semi-captive orangutans (Kleinschmidt et al., 2018; Labes et al., 2011). It is assumed that wild primates are infected with *S. fuelleborni*, while *S. stercoralis* is typical for captive animals, and is probably transmitted to primates from other susceptible hosts (Bradbury et al., 2021). A study providing exact *Strongyloides* species determination in free-ranging, semi-captive, and captive Bornean orangutans using ITS and 18S rDNA analysis revealed that free-ranging animals were infected with *S. fuelleborni*, while *S. stercoralis* was identified only in captive individuals (Labes et al., 2011). Moreover, Frias et al. (2018) found only *S. fuelleborni* in free-ranging Bornean orangutans in the Lower Kinabatangan Wildlife Sanctuary, Sabah, Malaysia. In contrast, *S. fuelleborni* has never been found in orangutans kept in zoological gardens. However, the transmission of *S. fuelleborni* between primates and animal caretakers in captive conditions has been demonstrated between Southern pig-tailed macaques (*Macaca nemestrina*) and their owners in Southern Thailand (Janwan et al., 2020) and transmission is also very possible in case of *S. stercoralis* between captive primates and the staff and *vice versa* (Bradbury et al., 2021).

In all orangutans from both Slovak and Czech zoos, *S. stercoralis* was detected by coproscopic detection of L1 larvae and amplification of the partial sequence of *cox1*, HVR-I, and HVR-IV regions of 18S rDNA confirmed its identity, which supported our hypothesis. Based on *cox1*, two lineages A and B of *S. stercoralis* are distinguished, with lineage A occurring in dogs, humans, and other primates, while lineage B has been recorded in dogs only (Bradbury et al., 2021). All *cox1* sequences obtained in the present study clustered within the lineage A (highlighted in dark blue in Figure 3). Similarly, only haplotype A of HVR-IV, so far detected in humans, chimpanzees, and dogs (Bradbury et al., 2021) was detected in our samples. In contrast, we found haplotypes II and VI of the HVR-I. Although the significance of the HVR-I is not yet known, our results indicate possible interspecific variability. In general, our results support the hypothesis that *S. stercoralis* occurs not only in dogs and humans, but also in

FIGURE 3 Phylogenetic tree derived by Bayesian inference and maximum likelihood for *Strongyloides* spp. *cox1* (722 bp) sequences obtained in this study (in bold) and available in the GenBank database. The tree was constructed from a muscle alignment and calculated using the GTR + G model for nucleotide substitutions. Branch lengths indicate expected numbers of substitutions per nucleotide site. Numbers at the nodes show posterior probabilities under the Bayesian inference/bootstrap support values for maximum likelihood. Nodal supports <50 are marked with an asterisk. A total of 18S rDNA haplotypes are marked in the tree.

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captive primates, whereas *S. fuelleborni* is restricted to free-ranging primates.

We detected zoonotic parasites with possible pathogenic potential, *S. stercoralis* and *B. coli*, in Bornean and Sumatran orangutans kept in several Czech and Slovak zoological gardens. Application of molecular, sequencing, and phylogenetic tools for exact identification of vestibuliferid ciliates is crucial as morphological distinction is difficult. We provided sequence data for *S. stercoralis* from captive orangutans and contributed to knowledge of the genetic diversity of the parasite as data from primates are very rare in public databases (Bradbury et al., 2021). Although none of the animals exhibited any clinical signs, these infections can lead, under some circumstances, to serious illness and even to death (Strait et al., 2012). Parasite monitoring is crucial for institutional health programs and reducing the risk of transmission to humans working with captive primates. Effective management, which includes monitoring of parasitic diseases, is a paramount component of the preventative medicine program of any facility that wants to reduce the risk of clinical illness in animals as well as the risk of zoonotic transmission (Sanchez & Pich, 2018). Regular parasite screening provides a base for targeted treatment, while regular and blind application of antiparasitic drugs can lead to resistance to the products and reduce their effect in the future, as known in livestock (e.g., Geurden et al., 2015).

ACKNOWLEDGMENTS

The authors thank all zoological gardens for providing the material, and especially to curators, zookeepers, and veterinarians who participated on the sample collection. We also thank the animal care staff at the CIRMF primate center for their help. This project was supported in part by the Japan Society for the Promotion of Science-Czech Academy of Science bilateral cooperative program, Japan/Czech Republic (project nos. JPJSBP120192506 and JSPS-19-17), by institutional support from the Institute of Vertebrate Biology, Czech Academy of Sciences, Czech Republic (RVO:68081766), by Marie Sklodowska-Curie Individual Fellowship (703611), and by institutional support from Masaryk University (MUNI/A/1488/2021).

CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

Data availability—sequences from *Strongyloides stercoralis*, *Balantioides coli*, and Buxtonella-like are available and uploaded to GenBank under the accession numbers OM392049–OM392052 (*cox1*); OM423701–OM423711 (ITS1-5.8s-rRNA-ITS2); OM423618–OM423632 (18S rDNA): see Supporting Information: Table S1.

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How to cite this article: Nosková, E., Modrý, D., Baláž, V., Červená, B., Jirků-Pomajbíková, K., Zechmeisterová, K., Leowski, C., Petrželková, K. J., Pšenková, I., Vodička, R., Kessler, S. E., Ngoubangoye, B., Setchell, J. M., & Pafčo, B. (2023). Identification of potentially zoonotic parasites in captive orangutans and semi-captive mandrills: Phylogeny and morphological comparison. *American Journal of Primatology*, 85, e23475. https://doi.org/10.1002/ajp.23475

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2026

Strongyloides genetic diversity among humans, dogs, and nonhuman primates, Central African Republic, 2016–2022.

Emerg. Infect. Dis. 32, 349–359. https://doi.org/10.3201/eid3203.250526

Strongyloides Genetic Diversity among Humans, Dogs, and Nonhuman Primates, Central African Republic, 2016–2022

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Strongyloides stercoralis nematode infection occurs in ≈600 million persons worldwide and is listed by the World Health Organization as a neglected tropical disease. Understanding zoonotic potential is critical, especially in areas where humans, domestic animals, and wildlife interact. We explored cross-species sharing of *Strongyloides* nematodes by analyzing fecal samples from humans, dogs, and nonhuman primates in the Dzanga-Sangha Protected Areas, Central African Republic. We detected positive samples by quantitative PCR and assessed genetic diversity through amplification of the 18S rRNA HVR-IV region and *cox1*, followed by high-throughput sequencing. *Strongyloides* prevalence was high in humans, dogs, and gorillas. *S. stercoralis* haplotype A nematode dominated in humans but appeared in dogs and apes, whereas *S. fuelleborni* nematode was present in all hosts. Shared species and haplotypes indicated zoonotic transmission. Our findings highlight the need for molecular surveillance and emphasize the role of dogs and nonhuman primates as reservoirs, complicating efforts to control infections in human populations.

Strongyloidiasis, caused by *Strongyloides* nematodes, is a zoonotic disease with public health and veterinary implications worldwide (*1*). The World Health Organization classifies strongyloidiasis among neglected tropical diseases requiring urgent control in endemic regions (*2*). Current estimates suggest that >600 million persons are infected globally, predominantly in tropical and subtropical areas (*3*). *Strongyloides stercoralis* and *S. fuelleborni* nematodes are the main species infecting humans; transmission typically occurs through transcutaneous exposure. More severe infections are primarily attributed to *S. stercoralis* nematodes, which are capable of autoinfection and can lead to severe systemic disease, particularly in immunocompromised persons; infection can result in death in extreme cases. Uncomplicated infections often manifest in gastrointestinal, pulmonary, or dermatological symptoms (*4*).

Molecular analyses of *S. stercoralis* nematodes have revealed 2 primary lineages: the potentially zoonotic lineage A, which involves dogs as potential reservoirs for human infection, and lineage B, which is largely restricted to canine hosts (*5*,*6*). Conversely, *S. fuelleborni* nematodes, although less common, are confined to nonhuman primates (NHPs) in Africa and Asia, occasionally spilling over to humans (*1*). Despite numerous documented cases, the true global prevalence of strongyloidiasis remains uncertain because of limited surveillance and underdiagnosis, compounded by the asymptomatic nature of many infections and the lack of standardized diagnostic tools (*7*,*8*).

The Central African Republic (CAR), one of the world's most resource-constrained countries (*9*), ranks 191st out of 193 on the 2022 United Nations Human Development Index (*10*). Its tropical ecosystems, which are rich in biodiversity and wildlife (*11*), create favorable conditions for zoonotic disease circulation (*12*). Frequent interactions

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DOI: https://doi.org/10.3201/eid3203.250526

among humans, NHPs, domestic animals, and wildlife encourage the spread of infectious diseases (*13*), including *Strongyloides* nematodes (*14*). Dogs often act as ecologic bridges as a result of their hunting and scavenging behaviors (*15*). Soil-transmitted helminth infections remain among the most prevalent public health challenges in CAR (*16*); *Strongyloides* nematodes pose a particular concern for both human and NHP populations (*14*,*17*).

This study investigates the role of potential animal reservoirs in human *Strongyloides* infections within the Dzanga-Sangha Protected Areas (DSPA), CAR, where humans, NHPs, and domestic dogs interact closely at the human–wildlife interface. By using molecular genotyping, we analyzed the diversity and sharing of *Strongyloides* haplotypes among humans, NHPs, and dogs to inform control strategies in complex multihost systems.

Materials and Methods

Study Design and Participants

The study was conducted in the DSPA in CAR (Figure 1) during 2016–2022, a location known for habituation of western lowland gorillas (*Gorilla gorilla gorilla*) to human presence and home of traditional BaAka hunter-gatherer communities living in close contact with wildlife. DSPA consists of several zones at varying levels of protection, including the strictly protected Dzanga-Ndoki National Park and the Dzanga-Sangha Special Reserve, a multiuse area where human activities are regulated to varying extents (Figure 1). The research complied with the legal requirements of the CAR and with all research, ethical, and sample transport approvals (Appendix 1, https://wwwnc.cdc.gov/EID/article/32/3/25-0526-App1.pdf).

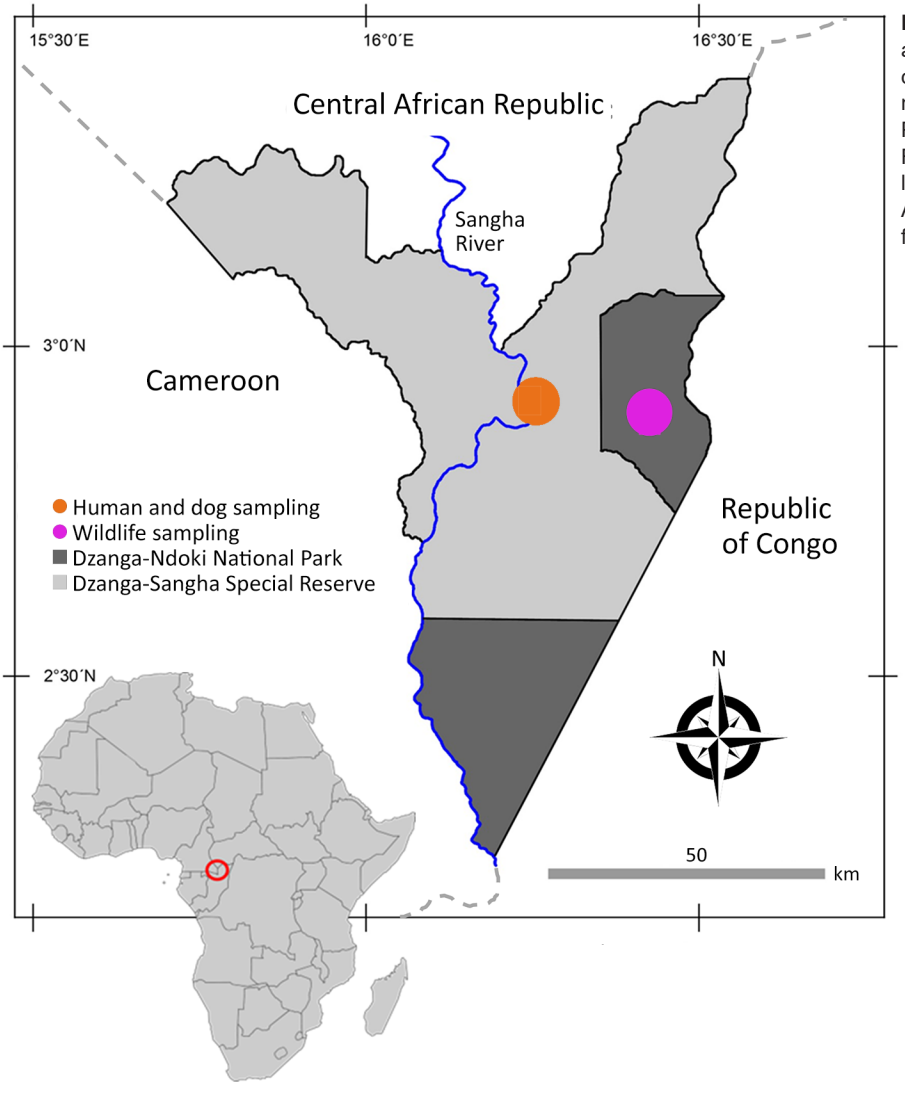


Figure 1. Location of study site in analysis of *Strongyloides* genetic diversity among humans, dogs, and nonhuman primates, Dzanga-Sangha Protected Areas, Central African Republic, 2016–2022. Inset shows location of Central African Republic in Africa. Figure adapted and adjusted from Hasegawa et al. (*18*).

Samples were collected from humans and domestic dogs from 2 villages located within the Special Reserve, whereas wildlife samples were collected in the National Park. Fresh stool samples were obtained from BaAka trackers who worked directly with NHPs in the National Park and also entered the Special Reserve (n = 18), as well as from humans residing in the villages (n = 32), who access the Special Reserve for daily activities such as gathering or hunting. Samples were also collected from dogs (n = 47), which were further categorized as hunting dogs entering the Special Reserve (n = 35) and guarding dogs (n = 12). Wildlife samples consisted of samples from western lowland gorillas (n = 101) at different levels of habituation, unhabituated central chimpanzees (*Pan troglodytes troglodytes*, n = 7), and a habituated group of agile mangabeys (*Cercocebus agilis*, n = 50). Samples were collected as part of health surveillance efforts (Appendix 2 Table 1, https://wwwnc.cdc.gov/EID/article/32/3/25-0526-App2.xlsx). Approximately 1 g of feces was collected and immediately fixed in 96% ethanol and stored at –20°C before DNA isolation.

Procedures

We dried fecal samples overnight at 37°C to evaporate the ethanol. We isolated total DNA using the PowerSoil DNA isolation kit (QIAGEN, https://www.qiagen.com). The extracted DNA was first screened by quantitative PCR (qPCR) targeting the small subunit (18S) rRNA gene specific to the genus *Strongyloides* (*19*) using the LightCycler 480 Real-Time PCR system (Roche, https://www.roche.com). We only included samples positive for *Strongyloides* in high-throughput library preparation. We used DNA extractions from *Strongyloides* PCR-negative feces and water as negative controls, whereas *S. stercoralis* first-stage larva was the positive control. All samples were prepared in technical PCR replicates (*20*). We prepared the high-throughput sequencing library using a 3-step PCR approach (Appendix 2 Table 2). We designed primers for the first PCR step, nested for the hypervariable region IV (HVR-IV) of the 18S rRNA gene and seminested for a portion of the mitochondrial cytochrome c oxidase subunit 1 gene (*cox1*), to increase sensitivity and ensure sufficient yield of amplicons for downstream sequencing (Appendix 1). In the second PCR step, we amplified the HVR-IV-18S rRNA and *cox1* (*21*). In the final PCR step, we applied Nextera primers with unique barcodes for each technical PCR replicate. We performed paired-end sequencing (2 × 300 bp) on the MGI DNBSEQ-G400 platform (MGI Tech, https://mgi-tech.eu).

We first demultiplexed raw fastq sequences and trimmed primer sequences using skewer version 0.2.2 (*22*). We then filtered, dereplicated, and denoised trimmed sequences and merged paired reads in R version 4.2.2 using the dada2 package (*23*). After processing, the final amplicon lengths were 255 bp for the 18S rRNA region and 217 bp for the *cox1*. During merging, we marked amplicon sequencing variants (ASVs) inconsistently present in both PCR technical replicates as potential artifacts and removed them from downstream analyses. We searched for corresponding sequences against the National Center for Biotechnology Information Nucleotide database (downloaded in February 2024) and excluded environmental, uncultured, <85% identity, and <90% coverage hits. We downloaded taxonomy using taxonomizer and used the created reference database to assign a taxonomic classification in our dataset through dada2's Assign Taxonomy method (*24*).

Statistical Analysis

We analyzed all data using the statistical software RStudio (https://www.rstudio.com). We assessed statistical differences in *Strongyloides* occurrence between trackers and villagers and between hunting and guard dogs using the χ^2 test. We generated a bar plot to depict the proportion of *Strongyloides* haplotypes of HVR-IV-18S rRNA in each sample for better resolution. For *cox1* haplotypes, we constructed a median-joining network and visualized using Population Analysis with Reticulate Trees (PopART, https://popart.maths.otago.ac.nz). In addition, we performed a phylogenetic analysis using the MrBayes plugin in Geneious 9.1.5 (https://www.geneious.com) to complement the network approach and assess the phylogenetic placement of the detected haplotypes. To examine differences in α diversity, assessed as ASVs richness (number of ASVs per sample), we applied a generalized linear model (GLM) with a quasipoisson error distribution. We conducted posthoc pairwise comparisons using the Tukey test to identify specific differences among the studied host groups. We evaluated community composition diversity by analyzing the relative representation of HVR-IV-18S rRNA and *cox1* ASVs using Bray-Curtis ecologic distances. We visualized the clustering patterns with principal coordinate analysis (PCoA). To test for interspecific differences in *Strongyloides* nematode community composition among hosts, we performed a permutational analysis of variance (PERMANOVA), followed by similarity analysis (ANOSIM).

Results

Strongyloides qPCR Detection

The number of *Strongyloides*-positive samples by qPCR was high across all studied host species; rates were 76% (38/50) in humans, 60% (28/47) in dogs, 59% (58/101) in gorillas, 43% (3/7) in chimpanzees, and 38% (19/50) in mangabeys. Among humans, BaAka gorilla trackers exhibited a slightly higher, although nonsignificant ($\chi^2 = 0.83$; p = 0.36), prevalence of infection (83.3% [15/18]) than that observed in villagers (71.9% [23/32]). Similarly, hunting dogs, which actively venture into the forest, showed a significantly ($\chi^2 = 12.32$; p = 0.0004) higher prevalence (74.3% [26/35]) than did guard dogs, which remain in villages (16.7% [2/12]). All qPCR-positive samples were further successfully sequenced with both targets (HVR-IV-18S rRNA and *cox1*), except for 3 mangabey samples that were near the detection threshold (cycle threshold ≈33–34), which likely explains their sequencing failure.

Strongyloides HVR-IV-18S rRNA Diversity

Genotyping identified a total of 11,472,181 high-quality HVR-IV-18S rRNA reads, with a median sequencing depth of 74,200 (range 1,221–302,518) per sample. Taxonomic analysis identified a total of 24 ASVs (Table). Six clustered into already known *Strongyloides* haplotypes: haplotype A of *S. stercoralis* and haplotypes K, L, M, P, and T of *S. fuelleborni*. Five *S. fuelleborni* ASVs did not match with any previously described haplotypes. Those ASVs were detected in only 9 samples (9/140 [6.4%]). Three haplotypes were host-specific (detected exclusively in human, dog, or gorilla), whereas 2 haplotypes were shared (1 between gorilla and mangabey and the other between human and dog). Six ASVs were classified as uncharacterized *Strongyloides* species and were found in 14 samples (14/140 [10%]). Three ASVs were detected in a single host, each occurring exclusively in mangabeys, whereas the remaining 3 were shared (2 between humans and dogs, and 1 within mangabeys). Last, 7 unassigned ASVs, detected in 19% of the samples, were tentatively classified as being most closely related to the order Rhabditida.

Overall, *S. fuelleborni* nematodes dominated across all studied hosts; haplotype L was the most prevalent variant (93.8% of total prevalence). Potentially zoonotic haplotype A of *S. stercoralis* was detected in 29% of samples from humans, dogs, and gorillas (Table). Relative abundances bar plot of *Strongyloides* HVR-IV-18S rRNA ASVs shows interspecific differences in *Strongyloides* community composition depending on host species (Figure 2). Humans were predominantly infected with *S. fuelleborni* haplotype L and *S. stercoralis* haplotype A (Table; Figure 2, panel A). *S. stercoralis* haplotype A was detected less frequently in BaAka gorilla trackers (67%) than in their village-dwelling relatives (78%), although that difference was not statistically significant ($\chi^2 = 0.63$; p = 0.43); haplotype A was absent in guard dogs but present in 30% of hunting dogs. NHPs were mainly infected with *S. fuelleborni* haplotype L. Unclassified *S. fuelleborni* and other unassigned *Strongyloides* variants were predominantly found in mangabeys (Table).

Zoonotic Potential of *Strongyloides* Nematode on the Basis of HVR-IV-18S rRNA

The highest number of *Strongyloides* HVR-IV-18S rRNA ASVs (8) was shared between humans and dogs, accounting for 33.3% of all observed ASVs. Those included haplotype A of *S. stercoralis*, haplotypes K, L, M, and P of *S. fuelleborni*, 1 unclassified *S. fuelleborni* (ASV_19), and 2 unidentified *Strongyloides* spp. (ASV_14 and ASV_21). Four ASVs (16.6% of all observed ASVs), haplotype A of *S. stercoralis,* and haplotypes K, L, and P of *S. fuelleborni* were shared between humans and NHPs. Haplotype A of *S. stercoralis* and haplotypes K, L, and P of *S. fuelleborni* were shared across humans, dogs, and gorillas (Figure 2, panel A).

Differences in *Strongyloides* HVR-IV-18S rRNA Communities

The number of ASVs per sample did not differ significantly among host species, as determined by a GLM (p>0.1) and subsequent Tukey test (Figure 2, panel B). However, the PCoA diagram based on

Table. Prevalence of individual *Strongyloides* hypervariable region IV 18S rRNA haplotypes assessed from quantitative PCR–positive samples across host species in study of *Strongyloides* genetic diversity, Central African Republic, 2016–2022

	No. (%)				
Haplotype	Humans, n = 38	Dogs, n = 28	Gorillas, n = 58	Chimpanzees, n = 3	Mangabeys, n = 16
S. stercoralis A	28 (73.7)	7 (25)	7 (12.1)	0	0
S. fuelleborni K	9 (23.7)	6 (21.4)	4 (6.9)	0	0
S. fuelleborni L	38 (100)	26 (92.8)	54 (93.1)	3 (100)	13 (81.3)
S. fuelleborni M	7 (18.4)	9 (32.1)	0	0	0
S. fuelleborni P	1 (2.6)	1 (3.6)	38 (65.5)	3 (100)	10 (62.5)
S. fuelleborni T	1 (2.6)	0	0	0	0
S. fuelleborni unclassified	2 (5.3)	3 (10.7)	2 (3.4)	0	2 (12.5)
Strongyloides unclassified	3 (7.9)	4 (14.3)	3 (5.2)	0	4 (25)

Bray-Curtis ecologic distances revealed distinct differences in *Strongyloides* community composition among humans, dogs, and NHPs (Figure 2, panel D). Those differences were further statistically confirmed by PERMANOVA ($F_{(4,137)} = 11.372$; $p = 0.001$) and ANOSIM ($R = 0.3435$; $p = 0.001$) tests. The overlap in *Stron-*

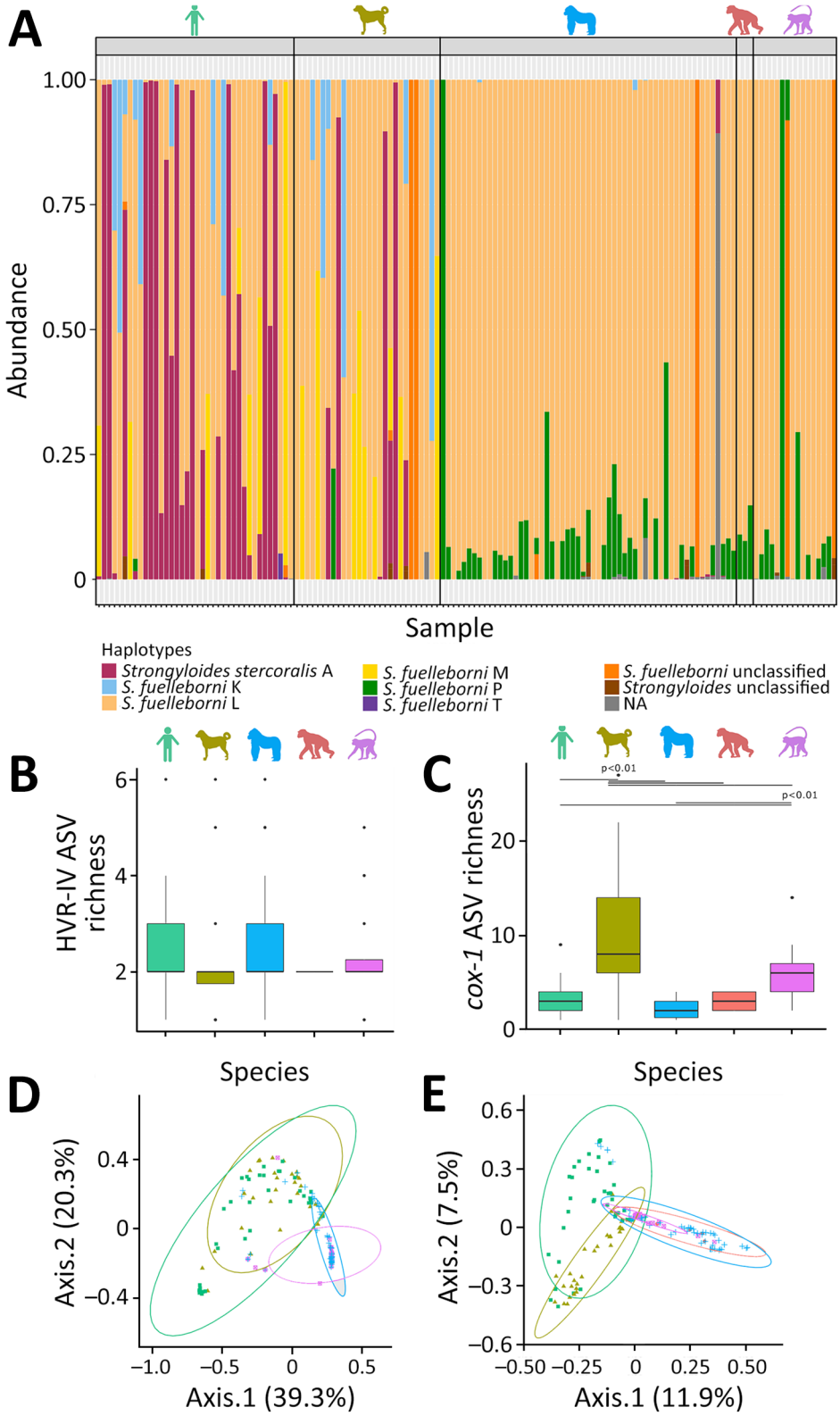


Figure 2. Relative community composition of *Strongyloides* HVR-IV-18S rRNA haplotypes across examined hosts in study of *Strongyloides* genetic diversity among humans, dogs, and nonhuman primates, Dzanga-Sangha Protected Areas, Central African Republic, 2016–2022. A) Relative abundance of haplotypes shown as color panels; each column represents a single sample. B) Boxplot showing the α diversity of *Strongyloides* HVR-IV 18S rRNA haplotypes, represented by the number of ASVs per sample (dots) grouped by host species. C) Boxplot showing the α diversity of *Strongyloides cox1* haplotypes represented by the number of ASVs per sample (dots) grouped by host species. D) Beta diversity of *Strongyloides* HVR-IV 18S rRNA haplotype communities, based on Bray-Curtis ecologic distances (relative abundance of reads), visualized using principal coordinate analysis ordination diagrams. E) Principal coordinate analysis showing the β diversity of *Strongyloides cox1* haplotypes. Color silhouettes (A-C) and data points (D, E) indicate host species: light green, human; olive green, dog; blue, gorilla; red, chimpanzee; violet, mangabey. ASV, amplicon sequencing variant; HVR-IV, hypervariable region IV.

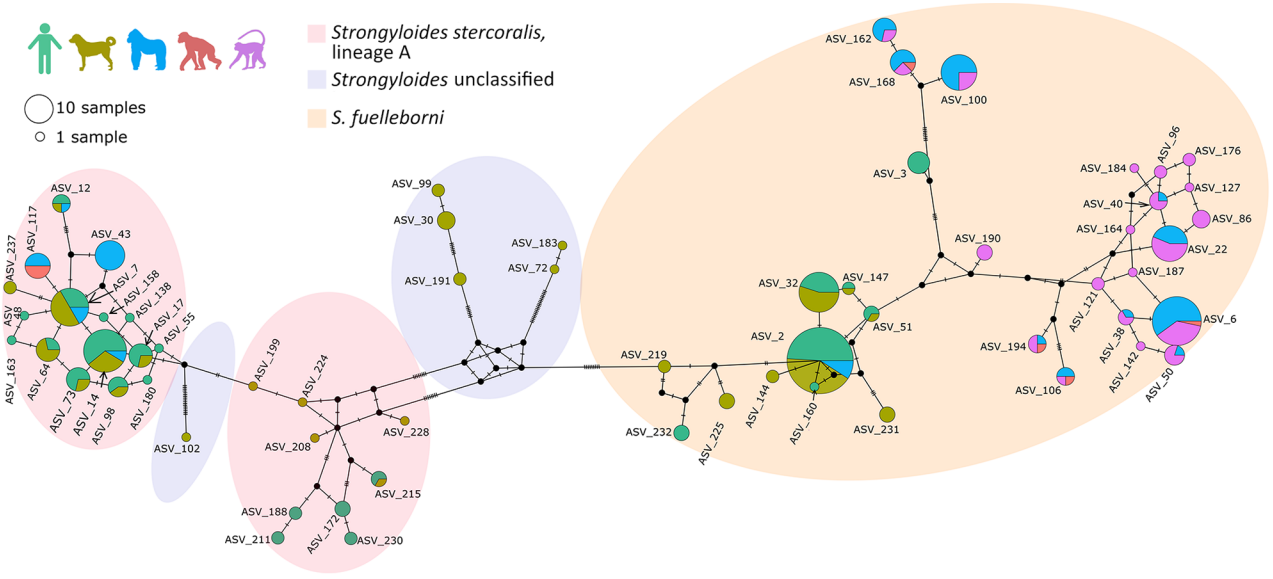


Figure 3. Median-joining *Strongyloides* haplotype network for the mitochondrial *cox1* gene studied in various host species in study of *Strongyloides* genetic diversity among humans, dogs, and nonhuman primates, Dzanga-Sangha Protected Areas, Central African Republic, 2016–2022. Each circle represents 1 haplotype; circle size indicates the number of hosts harboring the respective haplotype. The colors inside the circles indicate the host species: light green, human; olive green, dog; blue, gorilla; red, chimpanzee; violet, mangabey. The hatch marks beside the branches indicate the number of mutation steps between the haplotypes. Missing haplotypes are indicated by small black circles. Shaded ovals indicate species/lineage type. ASV, amplicon sequencing variant.

gyloides communities was greater between humans and dogs, whereas communities within NHPs were more similar but distinct from those of humans and dogs (Figure 2, panel D).

Strongyloides cox1 Diversity and Zoonotic Potential

We identified a total of 3,381,999 reads based on *cox1*; the median sequencing depth was 2,314 (range 75–56,108) per sample. Taxonomic assignment revealed 62 *Strongyloides* ASVs and 72 unassigned variants, which we tentatively classified as closest to the order Rhabditida. The 62 *Strongyloides* ASVs were used to construct a median-joining phylogenetic network (Figure 3) and Bayesian phylogeny (Figure 4).

The nucleotide diversity was high (N = 0.076), and 52 of the ASVs were parsimony informative. The haplotype network consisted of 25 *S. stercoralis*, 31 *S. fuelleborni*, and 6 unclassified *Strongyloides* ASVs. The network revealed divergence in ASVs on the basis of host specificity. *S. stercoralis* ASVs were primarily shared between humans and dogs, with occasional overlap with gorillas. Haplotype ASV_7 was most common in dogs, whereas ASV_14 was most frequent in humans. Chimpanzees shared only 1 haplotype (ASV_117) with gorillas, and intriguingly, haplotype ASV_43 was found exclusively in gorillas (Figure 3). In the phylogenetic tree, all ASVs of *S. stercoralis* cluster within potentially zoonotic lineage A (Figure 4). *S. fuelleborni* ASVs all clustered to *S. fuelleborni* Africa clade (Figure 4) and displayed host-specific divergence (Figure 3). Humans shared *S. fuelleborni* haplotypes mostly with dogs and occasionally with gorillas, whereas other haplotypes were restricted to NHPs. The ASV_2 haplotype was most common in both humans and dogs, whereas ASV_6 was predominant in gorillas and mangabeys. Haplotypes of unclassified *Strongyloides* species were detected only in dogs (Figure 4). The first cluster, consisting of ASV_30, ASV_99, and ASV_191, formed a well-supported branch closely related to *S. venezuelensis*. The second cluster, represented by ASV_183 and ASV_72, grouped between *S. planiceps* and *S. ratti* but with low nodal support. The final lineage, ASV_102, formed a separate, also weakly supported branch positioned between *S. ratti*, *S. procyonis*, and unclassified *Strongyloides* spp. found in Bornean slow lorises. Overall, the median-joining phylogenetic network and Bayesian phylogeny were highly complementary, revealing host-associated divergence in *S. stercoralis* and *S. fuelleborni* and highlighting distinct clustering of unclassified *Strongyloides* taxa.

Differences in *Strongyloides cox1* Communities

cox1 ASV richness differed significantly among the studied hosts (GLM $F_{(4,134)} = 253.93$; $p<0.0001$). Tukey posthoc testing revealed that dogs differed signifi-

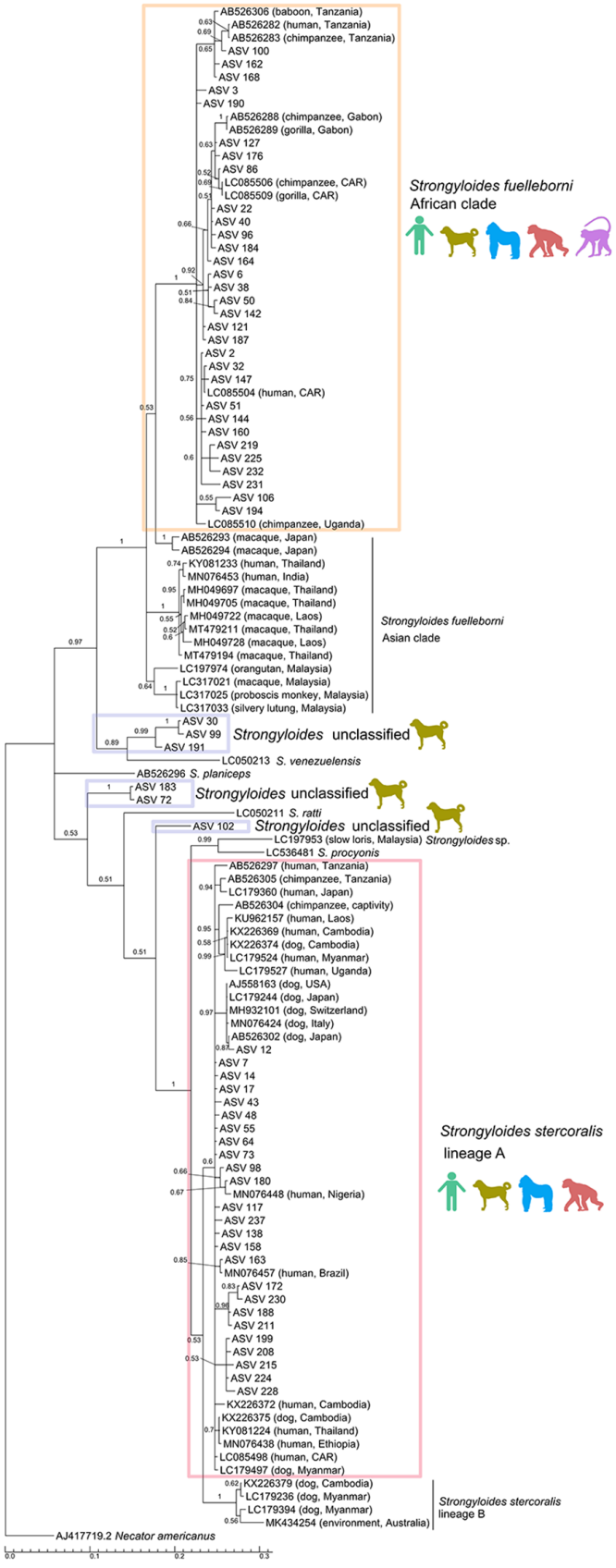


Figure 4. Bayesian phylogenetic tree based on *Strongyloides* spp. *cox1* (700 bp) sequences derived from study of *Strongyloides* genetic diversity among humans, dogs, and nonhuman primates, Dzanga-Sangha Protected Areas, Central African Republic, 2016–2022. Tree also includes sequences downloaded from GenBank; accession numbers are provided. The alignment was performed in Geneious (https://www.geneious.com) using the general time-reversible model of nucleotide substitution with gamma distribution. Branch lengths indicate expected substitutions per site. *Necator americanus* (accession no. AJ417719.2) was included as an outgroup. Node support was estimated from 10^6 iterations. Color silhouettes indicate host species: light green, human; olive green, dog; blue, gorilla; red, chimpanzee; violet, mangabey. Colored boxes indicate species/lineage type. ASV, amplicon sequencing variant; CAR, Central African Republic.

cantly from all other hosts ($p<0.01$ for all pairwise comparisons), whereas mangabeys also differed significantly from humans and gorillas ($p<0.01$) (Figure 2, panel C). We observed no significant differences between the remaining host pairs. The PCoA diagram based on Bray-Curtis ecologic distances further confirmed clear differences between humans, dogs, and NHPs (Figure 2, panel E), supporting the findings of the haplotype network analysis. Those differences in the composition of *Strongyloides* infections between host species were further statistically confirmed by PERMANOVA ($F_{(4,135)} = 11.192$; $p = 0.001$) and ANOSIM ($R = 0.3438$; $p = 0.001$) tests.

Discussion

We investigated *Strongyloides* nematode diversity and haplotype sharing among humans, dogs, and NHPs cohabiting in the DSPA, CAR, to assess zoonotic potential and multihost transmission (*25*). A previous study from the DSPA investigated *Strongyloides* nematodes in humans, gorillas, and chimpanzees, reporting *S. stercoralis* exclusively in humans and identifying different haplotypes of *S. fuelleborni* in gorillas and chimpanzees than those found in humans (*14*). However, that study was limited by a small sample size of larvae and did not include dogs, which are potential hosts. Our study highlights the critical importance of employing modern diagnostic approaches and examining a broader range of hosts from the same locality to better understand *Strongyloides* sharing in the ecosystem.

Our genotyping approach revealed a remarkable degree of genetic diversity within *Strongyloides* species, reflecting the complexity of their populations, and further identified the sharing of specific haplotypes across different host species. Of note, *S. fuelleborni*, likely originating from NHPs, dominated across all hosts; haplotype L was the most prevalent variant. Zoonotic *S. stercoralis* haplotype A was observed primarily in humans, dogs, and gorillas. This variation in prevalence underscores the host-specific transmission dynamics and the role of local ecologic factors in determining *Strongyloides* distribution. Those findings emphasize the need for a One Health approach (*26*) because of the zoonotic potential of *S. stercoralis* nematodes and the species' strong connection to the environment.

Despite the identification of dominant haplotypes, the proportion of *Strongyloides* sequences remains unclassified, highlighting the limitations of short-marker–based phylogenetic inference. Unclassified *S. fuelleborni* haplotypes were detected in all host species except chimpanzees, for which only a limited dataset was available. Those sequences likely reflect previously undetected diversity, because additional haplotypes have been reported with expanded sampling. Given the limited data currently available from sites in Africa (*27*), further haplotypes are expected to emerge as sampling across hosts and regions improves.

Several unclassified ASVs clustered with *S. venezuelensis* with high nodal support, whereas other unclassified ASVs formed clusters with very low support, rendering their classification unreliable. The placement of such lineages, based solely on short *cox1* fragments, is highly challenging, because those sequences might lack sufficient phylogenetically informative sites to resolve relationships among closely related or cryptic taxa. Similar issues have been reported previously (e.g., short *cox1* fragments were insufficient for precise taxonomic resolution of cryptic *Strongyloides* in dogs [*28*]). Therefore, integrating comprehensive genetic data, such as longer mitochondrial sequences or genomic information from individual larvae, with morphological analyses of larvae and adult or paratenic females is crucial for resolving the taxonomy and evolutionary relationships of unclassified *Strongyloides* spp.

We recognize the inherent limitations of single-locus genotyping, because reliance on a single marker provides a restricted representation of genetic diversity and might not reflect genome-wide variation. That limitation means substantially larger host sample sizes are required to achieve the same statistical power and robustness that whole-genome data can provide, because only by averaging across many individuals can stochastical effects at a single locus be mitigated. We also acknowledge that our reliance on fecal DNA, although advantageous as a noninvasive sampling method, can introduce issues related to DNA degradation, contamination, and allelic dropout, further constraining data quality. Thus, our conclusions should be interpreted with caution and viewed as complementary to, rather than a substitute for, genome-wide analyses.

Strongyloides communities were more similar between humans and dogs than between either of those hosts and NHPs. The highest proportion of shared haplotypes was observed between humans and dogs, accounting for 33.3% of all detected ASVs. This pattern supports the involvement of dogs in the circulation of *S. stercoralis* nematode alongside humans in the DSPA (*5*,*6*). Consistent with this pattern, *S. stercoralis* haplotype A predominated in humans, suggesting that human-associated circulation represents a key component of *Strongyloides* nematode occur-

rence in this setting and that dogs potentially act as secondary hosts.

Unexpectedly, sharing between humans and dogs was also observed for *S. fuelleborni* haplotypes K and M, a species previously considered to circulate primarily in NHPs (*14*). The presence of haplotype M in both humans and dogs, with higher prevalence in dogs, is notable given its limited geographic reporting to date in humans, restricted to Senegal (*21*) and CAR (*14*). The high prevalence in dogs suggests a potential contribution to the environmental dissemination of *S. fuelleborni* nematodes within DSPA, although spurious infections associated with coprophagy cannot be excluded (*29*). Experimental infections of dogs with *S. fuelleborni* nematodes have been demonstrated (*30*), but natural infections remain unconfirmed, and fecal metabarcoding alone cannot resolve the definitive host status of dogs.

ASVs shared between humans and NHPs included *S. stercoralis* haplotype A and *S. fuelleborni* haplotypes K, L, and P, accounting for 16.6% of all observed ASVs; all of those were also detected in dogs. *Strongyloides fuelleborni* haplotype L, which is well established across all studied hosts, was previously reported only in gorillas (*14*) and in African vervets on St. Kitts (*31*). Although its origin remains speculative, its presence across host species suggests extensive host sharing within the DSPA. In contrast, haplotype P was predominantly detected in NHPs, which likely represent a key source of exposure for humans and dogs. Together, these patterns indicate shared circulation of *Strongyloides* nematodes among humans, dogs, and NHPs in the DSPA.

Given the soil-transmitted nature of *Strongyloides* nematodes and the close coexistence of hosts in DSPA, environmental exposure represents a major challenge for disease control (*4*). Although chemotherapy is effective in the short term to treat strongyloidiasis (*32*), high reinfection rates, autoinfection, free-living stages in soil, multiple host species, and emerging concerns about drug resistance complicate long-term elimination, particularly in tropical settings (*33*,*34*), highlighting the need for a comprehensive strategy. A crucial aspect of controlling strongyloidiasis is recognizing the role of wildlife in transmission. Because treating wildlife such as free-ranging NHPs is not feasible, effective control requires a One Health approach that considers humans, domestic animals, and the environment (*26*). Practical measures, including proper latrine use and use of footwear to reduce soil contact, should complement treatment of infected persons (*35*). Ivermectin remains the treatment of choice (*33*), but its use must be carefully considered in regions with high *Loa loa* microfilaremia prevalence (*36*), as in CAR. Overall, the potential for rapid reinfection from multiple hosts underscores the need for integrated control strategies.

In conclusion, the close coexistence of multiple host species sharing *Strongyloides* infections in the DSPA likely contributes to the persistence of the parasite in this environment. Expanding investigations beyond humans to include domestic animals and wildlife is therefore essential, and our findings highlight the relevance of a One Health approach that integrates those host groups (*37*). The observed overlap of *S. stercoralis* and *S. fuelleborni* haplotypes among humans, dogs, and NHPs reflects the complexity of host associations within this ecosystem. By adopting a multihost diagnostic approach, we provide direct insights into *Strongyloides* nematode sharing among hosts in the DSPA, informing more realistic and sustainable control strategies.

Acknowledgments

We would like to express our gratitude to the government of the Central African Republic and the World Wildlife Fund for granting permission to conduct our research; the Ministre de l'Enseignement Supérieur, de la Recherche Scientifique et de l'Innovation Technologique, for providing research permits; and the Primate Habituation Programme for providing logistical support in the field. Finally, yet importantly, we would like to thank all the trackers and assistants in Dzanga-Sangha Protected Areas.

Raw HVR-IV-18S rRNA and *cox1* sequencing data are archived in the European Nucleotide Archive and are available project accession no. PRJEB101217. Accession numbers for each samples are available in Appendix 2 Table 3.

This project was supported by Czech Science Foundation 22-16475S and partially supported by Masaryk University internal grant no. MUNI/A/1602/2023. We acknowledge the CF Genomics supported by the NCMG research infrastructure (LM2023067 funded by MEYS CR) for their support with obtaining scientific data presented in this paper. Computational resources were provided by the e-INFRA CZ project (ID: 90254), supported by the Ministry of Education, Youth and Sports of the Czech Republic.

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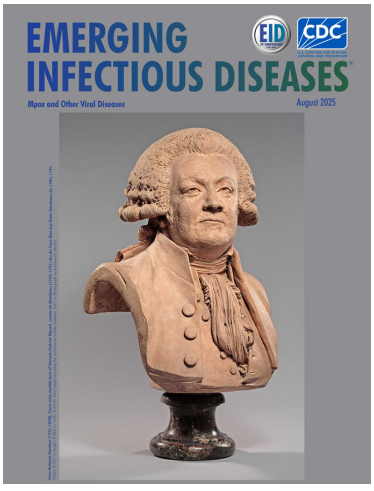
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2026

The potential threat of *Strongyloides* spp. to mountain gorillas and public health in Bwindi Impenetrable National Park, Uganda.

Parasitol. Res. 125, 52. https://doi.org/10.1007/s00436-026-08643-z

Parasitology Research (2026) 125:52
https://doi.org/10.1007/s00436-026-08643-z

RESEARCH

The potential threat of *Strongyloides* spp. to mountain gorillas and public health in Bwindi Impenetrable National Park, Uganda

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Received: 16 October 2025 / Accepted: 30 January 2026
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Abstract

Soil-transmitted nematodes *Strongyloides* spp. parasitize a range of vertebrates, including endangered great apes and humans. Since 2018, the Mountain Gorilla Veterinary Project has observed an increase in gastrointestinal parasitic infections, including suspected strongyloidiasis in mountain gorillas in Bwindi Impenetrable National Park (BINP), Uganda. To investigate the prevalence of *Strongyloides* and potential cross-host transmission, we conducted noninvasive fecal sampling of mountain gorillas, olive baboons, domestic dogs and pigs. Samples were examined by coproscopy and real-time PCR followed by sequencing of the HVR-IV 18S rRNA and *cox1* genes. *Strongyloides* prevalence was higher in mountain gorillas ranging near the park edge compared to forest interiors inside the park and among infant and juvenile mountain gorillas compared to other age groups. *Strongyloides fuelleborni*, a known zoonotic species, was identified in both baboons and mountain gorillas, suggesting sharing this particular parasite between the two primate species with potential implications for humans surrounding the park. In domestic animals, *S. ransomi* was detected in pigs, a zoonotic lineage *S. stercoralis* haplotype was detected in one dog, and another haplotype closely related to *S. ransomi* and *S. venezuelensis* was identified in another dog. The presence of zoonotic *S. fuelleborni* and *S. stercoralis* in Bwindi ecosystem poses a threat to mountain gorilla as well as human public health. Our study underscores the need for continued monitoring and community sensitization about the risk of cross-species transmission of these parasites.

Keywords Strongyloides · Public health · Mountain gorilla · Gastrointestinal parasites · Uganda · Zoonoses

Handling Editor: Julia Walochnik

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Published online: 18 February 2026

Introduction

Strongyloides spp. are soil-transmitted rhabditid nematodes that parasitize the gastrointestinal system of a variety of vertebrate hosts with cross-host transmission potential (Thamsborg et al. 2017). Among the ~60 known *Strongyloides* species, two – *S. stercoralis* and *S. fuelleborni* – infect humans as well as other primates, including great apes (Nutman 2017; Nosková et al. 2024). Since 2018, veterinarians from the Mountain Gorilla Veterinary Project (MGVP) have identified an increase in morbidity and mortality of mountain gorillas in Bwindi Impenetrable National Park (BINP) from severe gastrointestinal parasitic infections, a condition they have termed as chronic wasting (CW). Clinical symptoms of CW include emaciation, severe diarrhea, anemia, lethargy, browning of the hair coat, stunted growth, and in some instances, death. In several cases of CW in infant gorillas, high numbers (reaching thousands of eggs per gram of feces) of *Strongyloides* eggs have been observed (MGVP veterinary records) suggesting the potential role of these parasites in clinical illness.

Infection with *Strongyloides* spp. in humans ranges from light asymptomatic infections to chronic symptomatic strongyloidiasis and in rare events, particularly among immunocompromised individuals, life-threatening hyperinfection characterized by larval dissemination throughout organ systems (Buonfrate et al. 2013; Nutman 2017). In veterinary medicine, other *Strongyloides* spp. are associated with clinical disease mainly in young animals such as piglets, lambs, calves and foals (Deplazes et al. 2016; Thamsborg et al. 2017). However, captive non-human primates (NHP), including great apes, can develop severe strongyloidiasis from *S. stercoralis* infection with symptoms including acute lethargy, periodic dry cough, bloated abdomen, pneumonia, anemia, vomiting, and acute diarrhea (Leeflang and Markham 1986; Lowenstine et al. 2018). Except for primates, including humans, dogs are known hosts of *S. stercoralis*. Genetic analyses show two lineages, potentially species: lineage A occurring in dogs, humans, and other primates, though the transmission between dogs and humans is rare, and lineage B typically found in dogs (Barratt et al. 2019; Bradbury and Streit 2024; Nosková et al. 2024).

Globally, strongyloidiasis is considered a neglected tropical disease and estimated 300–600 million people are infected, with a high incidence recorded in both tropical and subtropical regions (Buonfrate et al. 2013; Puthiyakunnon et al. 2014; Beknazarova et al. 2016). In Africa, the overall prevalence of human *Strongyloides* infections is estimated to be between <1% and 1.60% (Odeniran and Ademola 2016; Eslahi et al. 2021), however this is likely an underestimate (Eslahi et al. 2021). Indeed, in Uganda, occasional local coproscopy-based studies have reported *S. stercoralis* prevalence ranging from 0.2% in a refugee camp in Mid-Western Uganda to up to 13% prevalence in fishing villages in Mukono, central Uganda (Lule et al. 2010; Nampijja et al. 2015; Morawski et al. 2017; Sanya et al. 2020). A recent study determined 8% prevalence of *S. stercoralis* in Mukono using multiplex real-time PCR (Natukunda et al. 2024). Finally, both *S. stercoralis* and *S. fuelleborni* have been detected in people living in the Bwindi Impenetrable National Park (Ashford et al. 1990). *Strongyloides* spp. have also been reported in Ugandan domestic animals including *S. avium* in poultry (Ssenyonga 1982), *S. ransomi* in pigs (Roesel et al. 2017; Ngwili et al. 2022), and unidentified *Strongyloides* species in goats (Gradé et al. 2007; Kalule et al. 2023) and various wildlife, such as ungulates (Guilbride et al. 1962; Apio et al. 2006; Fugazzola and Stancampiano 2012) or rodents in BINP (Mawanda et al. 2020). Finally, *Strongyloides* spp. were recorded from multiple NHP species, including mountain gorillas (Ashford et al. 1990; Kalema-Zikusoka et al. 2005) and olive baboons (Hope et al. 2004) in BINP, guenons from western Uganda (Gillespie et al. 2004), chimpanzees in Kibale and Bulindi forests (Muehlenbein 2005; Bezjian et al. 2008; Hasegawa et al. 2016; McLennan et al. 2017), and colobus monkeys in Kibale and Lake Nabugabo (Gillespie et al. 2005; Hodder and Chapman 2012). Of these studies, only Hasegawa et al. (2016) characterized the *Strongyloides* larvae as *S. fuelleborni* by nuclear and mitochondrial markers. Moreover, there are globally only a few epidemiological studies evaluating cross-host transmission of *S. stercoralis* (Hasegawa et al. 2016; Jaleta et al. 2017; Barratt et al. 2019; Beknazarova et al. 2019) and more are needed to determine the likelihood of transmission across different animal taxa.

The BINP is surrounded by a dense human population living in basic conditions typical for rural areas and some parts of the park have no buffer zones, facilitating interactions between humans and primates crossing the forest edge and park border (McNeilage et al. 2001; Mwima and McNeilage 2003; Seiler and Robbins 2016; Muylaert et al. 2021). Such conditions can enable transmission of pathogens, including *Strongyloides* (Schär et al. 2013; Muylaert et al. 2021) (Fig. 1). Given the potential of *Strongyloides* spp. to be a threat to human public health, as demonstrated by recently emerged clinical cases of strongyloidiasis in Uganda (Pecorella et al. 2022; Yahaya et al. 2023), as well as a threat to the endangered mountain gorillas, the aim of this study was to investigate the prevalence of these nematodes in mountain gorillas and other free-ranging wildlife and domestic animals around BINP. Furthermore, our goal was to evaluate sharing *Strongyloides* spp. among the studied hosts using both coproscopy and molecular techniques, which has not yet been done in this ecosystem.

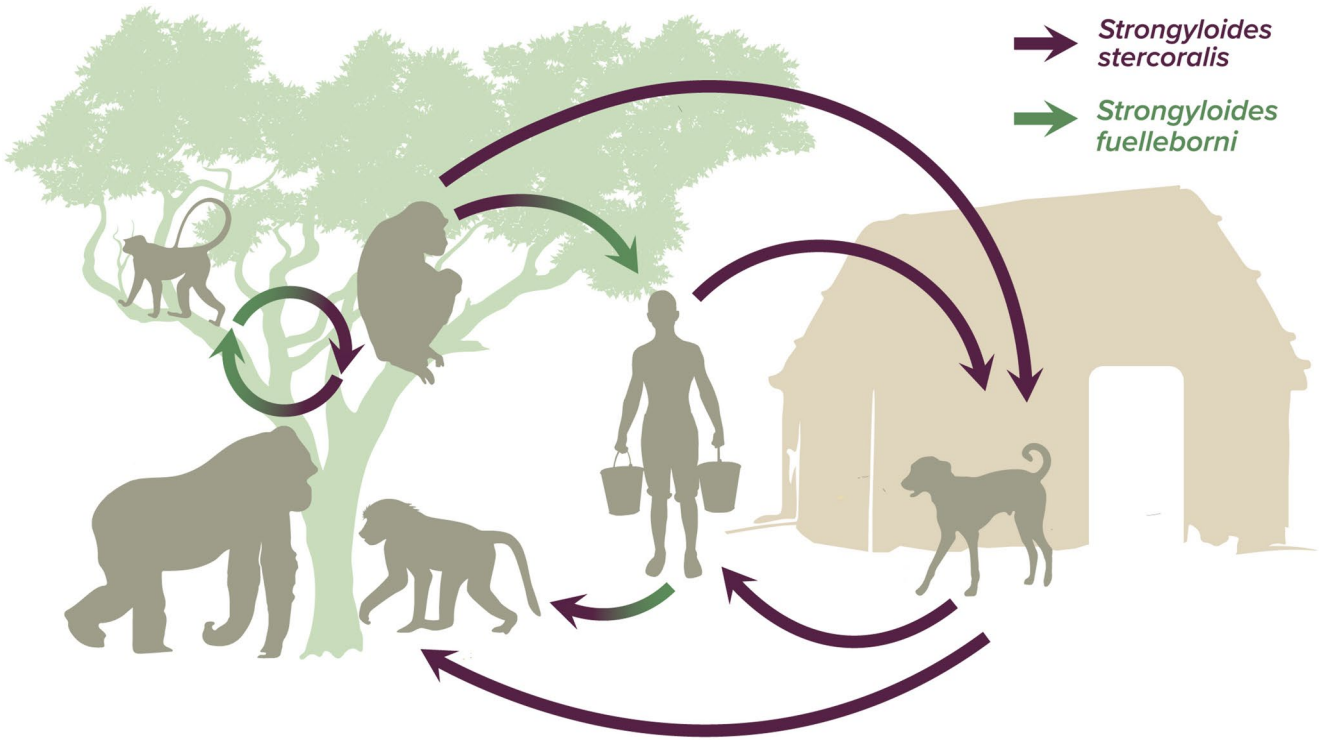


Fig. 1 Potential pathways for *Strongyloides* spp. transmission at the domestic-wildlife interface in Bwindi. Both species can circulate among non-human primates inside the forest, but humans can get infected by both species as well, while domestic dogs are known hosts of *S. stercoralis*. Baboons and dogs are often moving between the forest and community land and can easily spread the infectious stages in both environments

Materials and methods

Sampling and coproscopy

Targeted and opportunistic fecal samples were collected non-invasively from mountain gorillas ($N=141$), olive baboons ($N=27$), and dogs ($N=25$) around BINP between November 2021 and November 2022. Mountain gorilla samples were collected from the night nests after the animals had left. In olive baboons, the animals were followed and observed from a safe distance, and when defecation occurred, a sample was collected after the individuals moved away. Finally, feces from the dogs were collected after spontaneous defecation. Sex and age category of mountain gorillas, together with group affinity, were available for gorilla samples (Schaller 1963; Sinayitutse et al. 2021). Gorilla groups were further categorized based on their ranging patterns: those inhabiting areas near the forest edge and those residing deep within the forest interior. Non-invasively collected (from the pig pens) fecal samples from pigs ($N=110$) were opportunistically available from another study. In dogs and pigs, samples were collected with informed consent from the owners. Aliquots of fresh fecal samples were preserved in 10% formalin for coproscopic analyses and in 96% ethanol for molecular analyses. Fecal sediment was obtained by homogenizing, sieving and centrifuging (2000 RPM for 3 min) the formalin-preserved samples. Fecal sediment was weighed, and then all resuspended in 10 ml of 10% formalin. Finally, 1 ml of the resuspended fecal sediment was centrifuged (1500 RPM for 2 min), and the total sediment was transferred onto a slide for Strongyloides egg and larvae identification. Strongyloides eggs were quantified and recalculated as eggs per gram of fecal sediment (EPGS). Mountain gorilla samples were examined fresh by Mini-FLOTAC© as described in Petrželková et al. (2021) and quantified as eggs per gram of feces (EPGF).

Molecular methods

Ethanol preserved samples were used for DNA extraction using the DNeasy® Power Soil Pro Kit (Qiagen, Hilden, Germany) and subsequent detection of *Strongyloides* spp. DNA using probe based real-time PCR (qPCR) (Verweij et al. 2009). A subset of positive samples from each host species was used for 2-step PCR amplification of the hypervariable region IV of the 18S rRNA gene (HVR-IV; ~220 bp) and the cytochrome c oxidase 1 gene (*cox1;* 217 bp) as described by Nosková et al. (2026). Sanger sequencing was performed in both directions (Macrogen Europe B. V., Amsterdam, Netherlands). DNA sequences were manually

checked and trimmed in Geneious Prime® 2023.2.1 (www.geneious.com) followed by BLAST analysis. Obtained sequences were aligned with representative *Strongyloides* spp. sequences downloaded from GenBank and the resulting alignments were used to construct maximum likelihood phylogenetic trees by IQ-TREE (Trifinopoulos et al. 2016). The most suitable model was chosen by ModelFinder (Kalyaanamoorthy et al. 2017) implemented in IQ-TREE based on the highest Bayesian information criterion scores and weights. The tree topology was tested by 1000 replicates of ultrafast bootstrapping (Minh et al. 2013) and Shimodaira–Hasegawa (SH)-like approximate likelihood ratio test (Anisimova et al. 2011). The trees were visualized using the iTOL v6 software (Letunic and Bork 2024).

Statistics

Differences between the prevalence obtained by microscopy and qPCR (method effectiveness) calculated separately in each host were evaluated by McNemar's test in IBM SPSS Statistics (Version 20). For testing the differences in *Strongyloides* qPCR prevalence between the factors (group, forest position, sex/age category and sex), we used General Linear Models (GLM) with binomial distribution. For sex/age category, Tukey post-hoc tests were employed to test for differences among levels of factorial explanatory variables.

Results

Baboons had the highest *Strongyloides* prevalence and fecal sediment egg counts

Eggs corresponding to *Strongyloides* spp. were identified in baboon, pig, and mountain gorilla fecal samples. No eggs were found in dog samples, but a larva with morphology typical for *S. stercoralis* L1 was detected in one dog sample. Other observed gastrointestinal parasites included *Entamoeba* spp., *Trichuris* sp., *Buxtonella*-like ciliates and strongylid nematodes in baboons, *Trichuris* sp., strongylids, *Entamoeba* spp. and *Anoplocephala* sp. in mountain gorillas, *Ancylostoma* sp. in dogs and *Ascaris* sp., *Balantioides* sp., *Eimeria* spp. and *Trichuris* sp. in pigs. The highest *Strongyloides* egg counts and prevalence were observed in baboons compared to other host species (Table 1). Overall, there was a significant difference between the microscopic and qPCR methods, with qPCR detecting more *Strongyloides*-positive samples. The difference between the two methods was statistically significant in all host species except for the olive baboon (Table 1).

Age category and home range location affected *Strongyloides* prevalence

In mountain gorillas, the group affinity affected the qPCR prevalence of *Strongyloides* (GLM $p<0.001$), and prevalence ranged from 0% in Kyagurilo B group to 100% in the Nkuringo group. Any sex differences in *Strongyloides* prevalence were not observed (GLM $p=0.547$), but mountain gorillas in younger age groups were significantly more likely to test positive for *Strongyloides* [juveniles (GLM $p=0.047$), infants (GLM $p=0.004$)] compared to older gorillas (sub-adults, black-backs, adult females and silverbacks combined) (Table 2). Furthermore, mountain gorillas ranging nearby the forest edge were 2.14 times more likely to test positive for *Strongyloides* compared to gorillas residing deep in the forest interior (GLM $p=0.037$) (Table 2).

Baboons and gorillas shared *S. fuelleborni*

Sequencing of HVR-IV was performed on 10 mountain gorilla, 10 baboon, 8 pig, and 4 dog samples that tested positive by qPCR for *Strongyloides* spp. (Supplementary

Table 2 *Strongyloides* spp. prevalence by qPCR in mountain gorillas by sex/age group and location

Predictors	*n* sampled	*n* positive (%)
Sex/Age		
ADF	51	10 (19.6)
BB	13	7 (53.8)
INF	14	11 (78.6)
JUV	6	4 (66.7)
SAD	4	3 (75.0)
SB	13	4 (30.8)
Location		
Deep forest	42	13 (31.0)
Forest edge	59	26 (44.1)

ADF - adult female, *BB* - black back, *INF* - infant, *JUV* - juvenile, *SAD* - sub-adult, *SB* - silverback. In only 101 samples, sex/age category could be determined based on the fecal lobe size (Schaller 1963; Sinayitutse et al. 2021)

Table 1 Prevalence and fecal egg counts expressed as eggs per gram (EPG) of fecal sediment (baboons, pigs) or eggs per gram of feces (only in gorillas) together with McNemar's test comparing coproscopy and qPCR prevalence in different hosts

Hosts (sample size)	Number of positive samples in coproscopy	Mean EPG ± SD	Number of positive samples by qPCR	McNemar's chi-square	*p* value
Mountain gorilla (141)	3 (2.13%)	19.1 ± 224	70 (49.6%)	21.6	< 0.001
Olive baboon (27)	16 (59.3%)	116 ± 167	23 (85.2%)	3.6	0.06
Pigs (110)	9 (8.18%)	34.4 ± 120	34 (30.9%)	32.3	< 0.001
Dogs (25)	1 (4%)	1 L1	4 (16%)	12.9	0.0003

file S1). From the above samples, sequences obtained from gorillas ($n=6$, GenBank accession no. PX116722) and baboons ($n=6$, GenBank accession no. PX116721) were identical and showed 100% identity to *S. fuelleborni* isolates from chimpanzees in Bulindi, Uganda (GenBank accession nos. LC085491, LC085492, LC085493, LC085496), and a human in Dzanga Sangha Protected Areas (DSPA), Central African Republic (CAR) (GenBank accession no. LC085484). Three identical sequences of *S. ransomi* (GenBank accession no. PX116720) were obtained from pig samples, while two different haplotypes of *Strongyloides* were obtained from dogs (GenBank accession no. PX116718, PX116719): one showed 100% identity with *S. stercoralis* haplotype E *sensu* Barratt et al. (2019) (GenBank accession no. MK468674; from a dog in Australia) and the other showed 98.6% identity with various *S. ransomi* and *S. venezuelensis* isolates. In the phylogenetic tree (Fig. 2a), where sequences from gorillas, baboons, and pigs were represented by a single consensus sequence, our primate-originating sequences clustered in the African *S. fuelleborni* clade comprising all other sequences from various NHP as well as from humans. The Bwindi pig sequence clustered with other sequences of *S. ransomi* and *S. venezuelensis*. Sister to this clade was the sequence of *Strongyloides* sp. obtained from the dog sample D2, while the other sequence D12 clustered clearly in a *S. stercoralis* clade corresponding to the zoonotic lineage A *sensu* Barratt et al. (2019) comprising sequences from humans, NHP and dogs.

Lower success in amplifying and sequencing *cox1*

Although HVR-IV was successfully amplified in 17 out of 32 selected samples, *cox1* sequences of good quality were obtained only from 3 gorillas, 4 baboons, 3 pigs and 1 dog sample. Moreover, sequences obtained from pigs and a dog did not belong to any *Strongyloides* spp., but *Ascaris* and *Metastrongylus* in case of pigs or *Ancylostoma caninum* in case of the dog sample were amplified. Five more sequences from gorillas and baboons were of low quality, upon BLAST analysis, one sequence from a gorilla showed high similarity to a *Necator* sp., while the other four to *S. fuelleborni* (**Supplementary file S1**). Although the sequences from gorillas and baboons were of good quality, from 1 to 8 ambiguous nucleotides (double peaks) were detected in each of them, suggesting presence of multiple haplotypes in the sample. When aligned, these heterozygote nucleotides

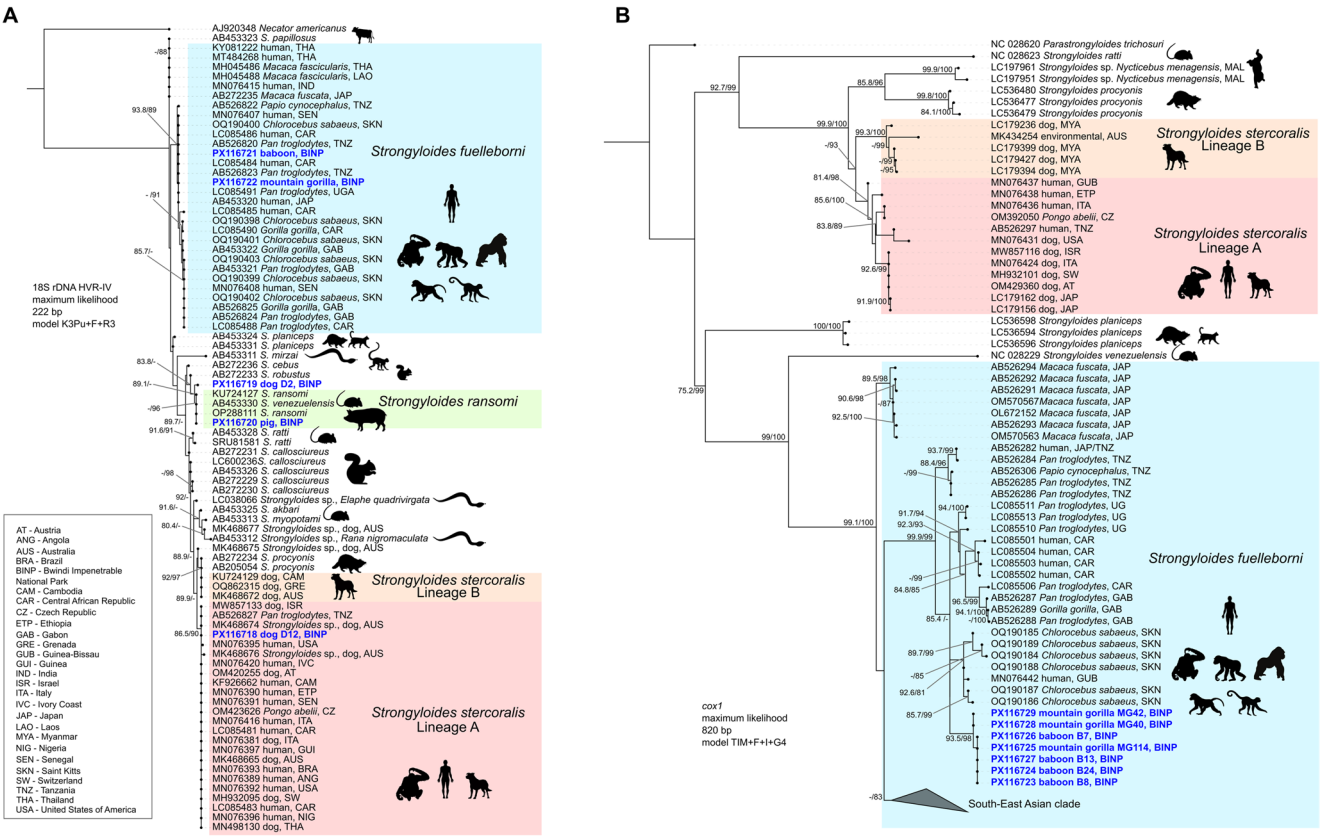


Fig. 2 Maximum likelihood phylogenetic tree of *Strongyloides* spp. **a**) 18S rDNA HVR-IV and **b**) *cox1*. Sequences from this study are in blue bold noting the host and code of the sample where relevant. Sequences downloaded from GenBank are labelled by the accession number and where relevant, host and country of origin. For other species, typical hosts are marked by the icons. Numbers at nodes are SH-aLRT support / ultrafast bootstrap support values, both in %. Only values above 80% are shown

occurred at 16 different positions of the alignment (269 bp). In the phylogenetic tree (Fig. 2b), all the Bwindi baboon and gorilla sequences clustered with other *S. fuelleborni* sequences from Africa, although they formed a separate sub-clade closely related to a clade comprising *S. fuelleborni* sequences from green monkeys in Saint Kitts Island, originally brought to the island from West Africa (Van Der Kuyl and Dekker 1996) and a sequence from a human from Guinea-Bissau (GenBank accession number MN076442).

Discussion

This study reported the prevalence of *Strongyloides* spp. in multiple hosts sharing the environment based on microscopy combined with molecular methods in the domestic animals and wildlife, including the mountain gorillas living in Bwindi, Uganda, and zoonotic species were identified in several hosts.

Overall, the qPCR detected higher numbers of positive animals compared to microscopy. In baboons, the results between qPCR and microscopy were similar, which is likely due to their comparatively high fecal egg counts. In mountain gorillas, there was a low number of shed eggs, which can be unevenly dispersed in feces and likely contributed to the substantial discrepancy between the number of positive samples detected by microscopy and qPCR. This combined with high numbers of strongylid eggs typically present in the Bwindi gorillas (Petrželková et al. 2021), makes low intensity *Strongyloides* infections more difficult to detect by microscopy, as the *Strongyloides* eggs might be overlooked. On the other hand, the used qPCR assay (Verweij et al. 2009) targets a conservative region of the 18S rDNA thus amplifying multiple *Strongyloides* species. However, as the primers are rather generic, further determination of the detected species is needed to correctly interpret the positive result.

Two commonly employed genetic markers were used to characterize *Strongyloides* spp. around the BINP: HVR-IV of the 18S rDNA and *cox1*. Both have been widely used in humans, NHP and dogs for sequencing of individual larvae or adult worms, and of recent, by metagenomics pipelines (Bradbury et al. 2021). Although the *cox1* gene is widely used as a standard marker for assessment of inter- and intraspecies diversity, and thus potential interspecies transmission, the HVR-IV is also effective for identification of individual *Strongyloides* species (Bradbury et al. 2021; de Ree et al. 2024).

In our study, the amplification and sequencing of the HVR-IV was successful in about half of the samples despite the faint bands in some of them. In contrast, *cox1* showed much lower amplification success rate and sequences contained ambiguous nucleotides suggesting presence of multiple haplotypes within an individual host, which was commonly observed in Australian dogs (Beknazarova et al. 2019) and St. Kitts vervets (Richins et al. 2023). In several cases, sequences were of low quality or other nematodes such as hookworms, lungworms or roundworms, were amplified instead of *Strongyloides.* This is not surprising as Barratt et al. (2019) reported off-target amplification of *Necator* and *Oesophagostomum* in humans using the same primers. On the other hand, the HVR-IV sequences showed only one haplotype in most host species and two haplotypes in dog. These findings are consistent with a previous metagenomic study from humans and dogs reporting multiple HVR-IV haplotypes in only a small percentage of samples (Beknazarova et al. 2019). However, a recent metagenomic study from St. Kitts showed presence of five to six *S. fuelleborni* HVR-IV haplotypes in all analyzed vervet samples (Richins et al. 2023). Therefore, both markers can help with determination of *Strongyloides* species, but the true genetic diversity of *Strongyloides* populations should be evaluated by metagenomic studies which allow for characterization of multiple haplotypes in a single sample.

The use of high-throughput sequencing approaches may also substantially improve the detection of *S. stercoralis*. Although we did not detect any *S. stercoralis* in gorillas or baboons using microscopy or Sanger sequencing, its presence cannot be excluded. The Mini-FLOTAC© method applied to gorilla samples is based on flotation and is therefore unsuitable for detecting *S. stercoralis*, which sheds L1 larvae rather than eggs. While the L1 larvae can, in principle, be detected in the sedimentation method used in baboons, larval stages are not as readily detected as *S. fuelleborni* eggs, particularly in formalin-preserved material where larvae are dead, shed in low numbers, and unevenly distributed (Uparanukraw et al. 1999). Standard microscopy diagnostic methods for *S. stercoralis* rely on active migration of L1 larvae, such as the Baermann larvoscopy or agar plate culture, which are substantially more sensitive than sedimentation techniques (Nosková et al. 2024; Watts et al. 2016). Consequently, failure to detect *S. stercoralis* by microscopy in this study may reflect methodological limitations rather than true absence.

Similarly, low larval output may explain why *S. stercoralis* DNA was not amplified or sequenced. Although both 18S HVR-IV and *cox1* primers used in this study have been previously shown to amplify both *S. stercoralis* and *S. fuelleborni*, amplification efficiency may differ between species. In samples dominated by *S. fuelleborni*, the more abundant taxon is likely to outcompete *S. stercoralis* in the early PCR cycles. As a result, the Sanger sequencing would preferentially recover the dominant taxon and thereby mask low-level *S. stercoralis* infections. High-throughput sequencing

would overcome this limitation by enabling detection of low-abundance haplotypes in mixed infections.

Finding *S. fuelleborni* eggs in mountain gorillas and baboons is consistent with previous studies that commonly reported this parasite in the two primate species across BINP, although the prevalence rates differed (Ashford et al. 1996; Hope et al. 2004; Kalema-Zikusoka et al. 2005; Rothman et al. 2006). While the overall microscopy-based prevalence for mountain gorillas found in this study was much lower than reported in the above-mentioned studies, the total qPCR-based prevalence reaching nearly 50%, with some gorilla groups having the prevalence even 100%, was higher than previously reported (Ashford et al. 1990, 1996). Furthermore, Ashford et al. (1996) detected higher *S. fuelleborni* prevalence in young animals which is consistent with our findings. Higher *Strongyloides* prevalence in young animals could be a consequence of the transmammary route of infection from infected breastfeeding mothers as known from dogs (Shoop et al. 2002), although the data on this route of transmission in primates is very scarce (Brown and Girardeau 1977), or to waning maternal immunity in combination with the naivety or immaturity of the infants' immune system.

On the other hand, while Ashford et al. (1996) and Kalema-Zikusoka et al. (2005) recorded egg counts ranging from 160 to 540 EPG and between 200 and 1200 EPG respectively, the values recorded in our study reached only tens of EPG. This could be a marker of improvements in the health of the general population due to veterinary clinical interventions on targeted individuals deemed fit for deworming under the MGVP clinical intervention policy. This approach has been highlighted by Robbins et al. (2011) as one of the advantages of extreme conservation with respect to endangered mountain gorillas.

Gorilla groups ranging at the edge of the forest and therefore the park border had significantly higher prevalence of *Strongyloides* than the groups residing inside the park. Baboons sampled in this study also live at the very edge of the forest, moving frequently between the community land and the park. These baboons had the highest prevalence, which was higher than previously reported by Hope et al. (2004), as well as the highest fecal egg counts among our studied hosts. The forest edge represents an important environment for pathogen transmission due to increased direct contact between people, their domestic animals and wildlife as well as environmental contamination by animal and human feces. Human legal or illegal activities at the forest edge such as wood collection or wire setting are often accompanied by poor sanitation as many villages close to the park lack permanent latrines (Creel et al. 2002; Chapman et al. 2006). High prevalence of *Strongyloides* spp. in both baboons and mountain gorilla groups ranging at the edge of the forest confirms an increased risk of *Strongyloides* infection in this shared environment. Moreover, detection of the same *S. fuelleborni* haplotype in both primate species suggests an ongoing cross-host transmission.

Several *Strongyloides* spp. have been detected in domestic dogs and pigs. Unsurprisingly, *S. ransomi* was confirmed in pigs, and a *Strongyloides* sp. closely related to *S. ransomi* and *S. venezuelensis*, typically occurring in rodents, was detected in one of the dog samples. The latter *Strongyloides* species is likely a case of spurious parasite, as most of the dogs around BINP are roaming free and their diet undoubtedly comprises scavenging and/or coprophagy (Goldsmid 1970; Campos et al. 2007). Since there is no known *Strongyloides* transmission between pigs and rodents or primates, the two taxa identified in pigs and a dog are unlikely to pose a threat to either mountain gorillas or humans. However, *S. stercoralis* HVR-IV haplotype E detected in the other dog sample corresponds to the zoonotic *cox1* lineage A occurring in both dogs and humans, although this specific haplotype has so far been reported in dogs only (Beknazarova et al. 2019). Nevertheless, Ashford et al. (1990) reported *S. stercoralis* larvae in humans in Bwindi, therefore the risk for human population is possible. Similarly, although rather rare in free-living NHP (Nosková et al. 2024), S. *stercoralis* may infect the mountain gorillas which can lead to serious health problems, especially in immunocompromised individuals (Penner 1981; Streit 2021).

This study provides the first genetic identification of *Strongyloides* spp. from mountain gorillas, baboons, dogs, and pigs around BINP. Our findings highlight an elevated risk of *Strongyloides* infection among mountain gorillas ranging close to the forest edge, likely due to shared environmental exposure and potential cross-species transmission with sympatric baboons. The detection of *S. stercoralis* Lineage A, a zoonotic genotype, in a local dog underscores the public health implications and raises concerns about possible transmission to humans and endangered great apes.

While previous studies have detected *Strongyloides* spp. around BINP through microscopy, (Ashford et al. 1996; Kalema-Zikusoka et al. 2005; Rothman et al. 2006) the use of molecular diagnostics provides stronger evidence for parasite identity and host overlap. Further research including sampling from people living in the park's proximity as well as utilization of metagenomics is needed to better understand transmission dynamics and the zoonotic potential of circulating *Strongyloides* strains around BINP. These findings emphasize the importance of adopting One Health-based interventions at the human-wildlife interface. Strategies such as reinforcing park boundaries, limiting the entry of humans and domestic animals, improving sanitation and community awareness are critical to reducing parasite transmission and safeguarding the health of both human populations and the critically endangered mountain gorillas.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s00436-026-08643-z.

Acknowledgements The Uganda Wildlife Authority (UWA) REF COD/96/05, the Uganda National Council for Science & Technology (UNCST) REF HS2416ES, the Veterinary Ethical Review Committee (VERC) REF 1.23 from the University of Edinburgh, and Makerere University (CEBIGH) laboratory.

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Funding Open access publishing supported by the institutions participating in the CzechELib Transformative Agreement. This work was financially supported by The Morris Animal Foundation (D21ZO-042), U.S. Fish & Wildlife Service (USFWS) – Great Ape Conservation Act (F22AP02231-00), Czech Science Foundation (22–16475S), Gorilla Doctors (MGVP), Society for Conservation Biology (SCB) - African Chapter, and The Gorilla Organization.

Declarations

Fecal samples from animals involved in this study were collected non-invasively without causing any disturbance to the animals. In the case of domestic animals, consent was obtained from the owner. The study was approved by the Veterinary Ethical Review Committee (VERC) REF 1.23 from the University of Edinburgh, Uganda Wildlife Authority (UWA) REF COD/96/05, and the Uganda National Council for Science & Technology (UNCST) REF HS2416ES.

Competing interests The authors declare no competing interests.

Data Sequences obtained in this study have been deposited in the GenBank under the accession numbers PX116718-722 (18S rDNA HVR-IV) and PX116723-729 (*cox1*).

Clinical trial number Not applicable.

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Upadhayay, P., Kváč, M., Sambucci, K.M., Nosková, E., Ilík, V., Pafčo, B., Holubová, N., Červená, B., Schoof, V.A.M.

2026

Overlap in parasite communities among vervet monkeys, humans, livestock and dogs at Lake Nabugabo, Uganda.

Parasitology 153, 796–812. https://doi.org/10.1017/S0031182026101978

Parasitology

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Overlap in parasite communities among vervet monkeys, humans, livestock and dogs at Lake Nabugabo, Uganda

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Research Article

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Cite this article: Upadhayay P, Kváč M, Sambucci KM, Nosková E, Ilík V, Pafčo B, Holubova N, Červená B, Schoof VAM (2026) Overlap in parasite communities among vervet monkeys, humans, livestock and dogs at Lake Nabugabo, Uganda. *Parasitology* **153**, 796–812. https://doi.org/10.1017/S0031182026101978

Received: 29 October 2025
Revised: 6 March 2026
Accepted: 5 April 2026
First published online: 17 April 2026

Keywords:
metabarcoding; nematodes; parasites; protists; sympatric hosts; zoonoses

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Abstract

Zoonotic parasites are a growing concern in regions where humans, domestic animals and wildlife live in close proximity. Understanding parasite communities overlap across hosts is essential for understanding transmission risks. In this study, we used sedimentation technique followed by microscopy, and molecular techniques for the identification of strongylid nematodes, *Strongyloides*, and protists in vervet monkeys (*Chlorocebus pygerythrus, N* = 75), household latrines (*N* = 25), dogs (*Canis lupus familiaris, N* = 25) and livestock ($N_{cows} = 9$, $N_{goats} = 7$, $N_{pigs} = 9$) at Lake Nabugabo, Uganda to compare parasite assemblages across hosts within the same habitat. We identified 33 unique parasite taxa with 18 in dogs, 14 in livestock and 10 each in vervets and latrine samples. There was a significant difference in the parasite infection rate between vervets (88%), latrines (24%), livestock (44%) and dogs (40%). Samples harboured at least 7 known genera of strongylid nematodes, with *Ancylostoma, Murshidia* and *Oesophagostomum* being the most prevalent. Protists diversity varied across hosts. Species-specific *Cryptosporidium* taxa occurred in dogs, goats (*Capra aegagrus hircus*) and pigs (*Sus scrofa domesticus)*, and *C. andersoni* was detected in both dogs (4%) and cows (*Bos taurus*, 8%). *Giardia intestinalis* assemblage A was found in vervets (1.3%), dogs (16%) and latrine samples (12%). *Enterocytozoon bieneusi* occurred only in latrine samples, while *Encephalitozoon* sp. occurred only in vervets (4%). Our results show limited overlap between vervets and other hosts but highlight shared *Giardia* among animals and latrine samples, indicating key points of potential zoonotic transmission in this human-modified landscape.

Introduction

It is estimated that over 60% of human infectious diseases are zoonotic in origin (Taylor et al., 2001). However, most disease control programmes focus on humans only (Morse et al., 2012), following single-pathogen single-host epidemiological theory (Antonovics, 2017). In the last few decades, there has been an increase in the interaction and exposure among humans, livestock and wildlife due to increased intrusion by humans into wildlife habitat, which has led to transmission and evolution of pathogens, and the emergence of infectious diseases (Cleaveland et al., 2001; Jones et al., 2008; Cole and Viney, 2018). Shared habitats create pathways for gastrointestinal parasite transmission, primarily via faecal-oral and soil-transmitted routes, as parasite stages are shed in faeces and contaminate soil and water sources used by multiple host species (Brooker et al., 2004; Cibot et al., 2015; Obanda et al., 2019). Helminths such as strongylid nematodes and *Strongyloides* are multicellular worms, so-called soil-transmitted nematodes, that develop through distinct egg and larval stages in the environment and usually do not multiply within a single host, with the exception of *S. stercoralis* that can hatch larvae within the host, initiate autoinfection and progressively increase the burden. On the other hand, unicellular eukaryotic parasites such as *Cryptosporidium, Giardia* and microsporidia transmitted via faecal-oral way replicate within host cells and are transmitted via environmentally resistant oo/cysts or spores (CDC, 2024a). However, both intrinsic (host immune response,

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physiology, etc.) and extrinsic (rainfall, temperature, etc.) factors contribute to determining whether a parasite eventually establishes itself within a particular host species or population (Turner and Elena, 2000; Duffy et al., 2007). While gastrointestinal parasites, including helminths and protozoans, are generally less fatal than many viral or bacterial pathogens, long-term infections can still cause significant health impacts and increase host morbidity (Alum et al., 2010).

Strongylid nematodes, a diverse group of gastrointestinal parasites, infect a wide range of mammalian hosts, including ruminants, carnivores, humans or non-human primates (Brooker et al., 2004; Van Wyk et al., 2004; Lichtenfels et al., 2008; Ilík et al., 2023). Although some strongylids exhibit certain host specificity, others are rather generalists and pose zoonotic risks in areas where the host species live in close proximity and share habitats and resources (Ghai et al., 2014; Hasegawa et al., 2014, 2017; Ota et al., 2015; Pafčo et al., 2019). For example, some strongylid nematodes in cattle such as *Ostertagia ostertagi, Haemonchus placei* or *Cooperia oncophora* (Charlier et al., 2012; Jabbar et al., 2014; Pafčo et al., 2024) or *Strongylus vulgaris* in equids (Jürgenschellert et al., 2022) show strong host specificity, while *Oesophagostomum* spp. infect broad range of hosts. Zoonotic potential of *O. bifurcum* and *O. stephanostomum* has been demonstrated by detection of these parasites in humans and other primates in Africa (Pafčo et al., 2019; Ilík et al., 2023), including in Uganda (Ghai et al., 2014; Cibot et al., 2015). Different *Oesophagostomum* spp. are commonly found in domestic animals such as ruminants or pigs (Sharma et al., 2011; Mishra et al., 2024). Typically, parasitic strongylid nematodes assemble into diverse communities that include multiple coexisting species and genera (Van Wyk et al., 2004; Lichtenfels et al., 2008; Mclean et al., 2012).

Strongyloides is a genus of nematodes with approximately 50 species of intestinal parasites that exclusively infect vertebrates such as amphibians, reptiles, birds and mammals (Viney and Lok, 2015). Infection caused by *Strongyloides* is also frequently documented in both free-ranging and captive primates across all continents except Antarctica, with a possibility of missing data from captive primates in Australia (Nosková et al., 2024). So far, 3 species have been described in primates; *Strongyloides cebus* has exclusively been identified in the Central and South American primates (Dorris et al., 2002; Mati et al., 2013), with no records of human infections with this species. While it is believed that *S. fuelleborni* is the primary cause of infection in wild non-human primates, there have been reports of mixed infections involving both *S. stercoralis* and *S. fuelleborni* in wild central chimpanzee (*Pan troglodytes troglodytes*) in Tanzania (Hasegawa et al., 2010), indicating that simultaneous infections may be relatively common (Hasegawa et al., 2016a). *Strongyloides fuelleborni* was found mainly in humans that shared habitat with other primates, while *S. stercoralis* has worldwide distribution in humans (Nosková et al., 2024). *Strongyloides stercoralis* can also infect dogs and cats (*Felis catus*) (Thamsborg et al., 2017). In experimental settings, *S. stercoralis* has been effectively transmitted from humans to dogs (Jariwala et al., 2017; Thamsborg et al., 2017). However, recent comparative research using mitochondrial *cox1* and nuclear 18S rDNA markers found the presence of 2 distinct genetic populations of *S. stercoralis* in dogs: type B seemingly exclusive to dogs, while type A is found in both dogs and primates including humans (Jaleta et al., 2017; Nagayasu et al., 2017). *Strongyloides* spp. are also found in domestic animals worldwide such as *S. westeri* in foals, *S. papillosus* in cattle and small ruminants, *S. ransomi* in pigs and so on (Thamsborg et al., 2017).

Cryptosporidium, Giardia and microsporidia are found in both humans and other animals; the infectious diseases caused by them (cryptosporidiosis, giardiasis and microsporidiosis respectively) have been documented in high-income countries as well as low- and middle-income countries (Fayer, 2010; Thellier and Breton, 2008; Xiao and Fayer, 2008). There are currently 48 recognized species of *Cryptosporidium* (Stensvold et al., 2024) with the literature describing over 150 genotypes, and in humans, at least 25 species and 3 genotypes have been detected, with *C. hominis* and *C. parvum* being the most common and responsible for diarrheal disease of varying severity (Fayer, 2010; Xiao, 2010; Stensvold et al., 2024). In cattle, the dominant species are *C. parvum, C. bovis, C. ryanae* and *C. andersoni*, with *C. parvum* as the major cause of clinical disease in young calves (Thomson et al., 2019). Goats commonly harbour *C. parvum, C. ubiquitum* and *C. xiaoi* (Ryan et al., 2021), while in pigs, *C. scrofarum* and *C. suis* are most frequently reported, with *C. parvum* also present in some countries (Chen et al., 2023). In dogs, *C. canis* is the primary species detected and can occasionally infect humans (Taghipour et al., 2020). In the 1920s, over 50 *Giardia* species were proposed, but current research focusing on trophozoite/cyst morphology acknowledges only 6 species (Ryan and Cacciò, 2013). *Giardia intestinalis* (syn. *G. lamblia, G. duodenalis*), the species infecting humans and most mammals, comprises 8 valid assemblages A–H, among which assemblages A and B are the primary causes of human giardiasis and are associated with diarrhoea, malabsorption and weight loss (Ryan and Cacciò, 2013; Mohammed, 2024). In non-human primates such as vervet monkeys, infections typically involve assemblages A and B, mirroring human pathogenic patterns (Feng and Xiao, 2011). In dogs, assemblages C and D are most common and are generally host-adapted with variable pathogenicity, while occasional infections with zoonotic assemblage A also occur (Thompson, 2004; Sprong et al., 2009). In livestock, assemblage E predominates in goats, pigs and cattle, usually causing subclinical to mild gastrointestinal disease, although diarrheal outbreaks have also been documented (Feng and Xiao, 2011; Ryan and Zahedi, 2019).

Certain genotypes of microsporidian *Enterocytozoon bieneusi*, such as genotypes B and Peru7, are primarily host-specific, whereas other genotypes such as D and EbpC are considered zoonotic and have been detected in both humans and other animals, demonstrating cross-species transmission potential (Thellier and Breton, 2008). *Cryptosporidium* spp., *G. intestinalis* and several microsporidia that can be transmitted between animals and humans have been reported in humans, livestock, companion animals and wildlife (Thellier and Breton, 2008; Xiao and Fayer, 2008; Ryan and Cacciò, 2013). Several studies have shown that non-human primates are potential sources of *Cryptosporidium, Giardia* and microsporidia infection for humans (Du et al., 2015); cases of human-pathogenic *Cryptosporidium* spp., *G. intestinalis* and *E. bieneusi* infections in wild, semi-wild and captive primates have also been reported in Asia, Africa and the Americas (Nizeyi et al., 2002; Ye et al., 2012, 2014; Sak et al., 2013, 2014; Karim et al., 2014; Mynářová et al., 2016) owing to the close phylogenetic relationship between humans and primates (Du et al., 2015).

To this end, the present study addresses 2 central research questions: (1) How do parasite prevalence and species richness differ among sympatric hosts, including humans, vervet monkeys, livestock and dogs, at Lake Nabugabo, Uganda? and (2) Which parasite species from the order Strongylida, and the genera *Strongyloides, Cryptosporidium, Giardia, Encephalitozoon* and *Enterocytozoon* are shared among these host species?

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Materials and methods

Study site

The study was conducted on the shores of Lake Nabugabo, Uganda (0°22′–12° S, 31°54′ E, 1136 m, Supplementary Figure 1), a satellite of Lake Victoria that is mostly surrounded by wetlands, grasslands, patches of degraded forest, farmers' fields and small buildings (Chapman et al., 2016). The area receives an average of 1348 mm of rain annually (Chapman et al., 2016) in 2 seasons with high rainfall (March–May and September–November) and 2 seasons with low rainfall (December–February and June–August) (Schwegel et al., 2023). The Nabugabo Research Site on the shores of the lake is home to long-term research in 3 neighbouring groups of vervet monkeys, which served as subjects for this study.

Sample collection

Between July and September 2022, 75 faecal samples (mean = 2.03, range = 1–4 per individual) from adult (>5 years or dispersal for males, after first birth for females) and subadult (4–5 years for males, 6 months before adulthood for females) vervet monkeys in 3 neighbouring groups were collected (25 samples from each group; $N_{male} = 30$, $N_{female} = 45$). We also non-invasively collected 25 faecal samples from different dogs, 25 individual faecal samples from different livestock (9 cows, 7 goats and 9 pigs), as well as samples from the 25 different pit latrines of households in Bbaale village nearby Lake Nabugabo, Uganda. The faecal samples from vervets, dogs and livestock were collected as soon as the individuals were observed defecating to avoid contamination with the environment/soil. The samples from the pit latrines were collected using a sludge nabber, which was properly washed and disinfected with 90% ethanol after each sample collection. All collected samples were fixed in 10% formalin for microscopic analysis and 96% ethanol for molecular analysis. The samples were kept at room temperature until they were transported to the labs for the analyses.

Microscopic analysis

The samples fixed in 10% formalin were analysed at the Central Diagnostic Laboratory, College of Veterinary Medicine Animal Resources and Biosecurity, Makerere University, Kampala, Uganda using formalin-ether sedimentation technique (Uga, 2010). The parasites were identified to the family and genus level wherever possible (Soulsby, 1982; Zajac and Conboy, 2006).

DNA isolation and sequencing

Total faecal DNA was extracted for detection of strongylid nematodes and genus *Strongyloides* from ethanol-preserved faecal samples using the DNeasy PowerSoil Pro kit (Qiagen, Germany) after the aliquots were left overnight at 37 °C to evaporate the residual ethanol at the Institute of Vertebrate Biology, Czech Academy of Sciences. No modifications were made to the manufacturer's protocol. Eluted DNA was stored at −20 °C until further analyses.

The internal transcribed spacer 2 (ITS2) of strongylid nematodes was amplified using primers Strongyl_ITS-2_F and Strongyl_ITS-2_R and high-throughput sequencing (HTS) (Pafčo et al., 2018, 2019). Briefly, the HTS libraries were generated using a 2-step-PCR approach following the Nextera primer design. DNA extractions from strongylid negative human faeces and water were used as negative controls and synthetic DNA templates as positive controls. All samples were prepared in 2 technical PCR replicates with different tag primer barcodes. The final library was sequenced using MiSeq Reagent Kit v3 (2 × 300 bp pair-end reads, 600 cycles) by Illumina MiSeq platform.

Strongyloides spp. DNA was detected by real-time quantitative PCR (qPCR) at the Real-Time PCR LightCycler® (Roche, Switzerland) targeting a conservative region (101 bp) of the gene for 18S rRNA (18S rDNA) using primers Stro18S-1530F, Stro18S-1630R and the probe Stro-18S-1586T (Verweij et al., 2009). A subset of positive samples from each host species was then used for amplification of the hypervariable region IV of 18S rDNA (HVR-IV; ∼255 bp) in a conventional PCR assay using primers New_HVR-IV_F and New_HVR-IV_R (Barratt et al., 2019). The PCR amplification and subsequent product treatment were carried out as described previously (Nosková et al., 2026). Purified products were sent for commercial sequencing to Macrogene (Macrogene Europe, Netherlands).

Total genomic DNA (gDNA) for detection of *Cryptosporidium* spp., *G. intestinalis, E. bieneusi* and *Encephalitozoon* spp. was isolated using the GeneAll® Exgene™ Stool DNA Mini Kit (GeneAll Biotechnology Co., Ltd, South Korea) according to the manufacturer's guidelines, with the following modifications at the Institute of Parasitology, Biology Centre, Czech Academy of Sciences. The alcohol was evaporated from each sample at 65 °C overnight. A 100 µL of 0.5 mm glass beads and 1000 µL of lysis buffer were added to each tube and oo/cysts/spores were disrupted for 60 sec at 5.5 m s^{-1} using glass beads in a FastPrep®-24 Instrument (MP Biomedicals, USA). The extracted gDNA was then stored at −80 °C. Nested PCR protocols were used to identify *Cryptosporidium* spp. at partial sequences of gene encoding small subunit of rRNA (18S rDNA), *G. intestinalis* at partial sequences of gene encoding triosephosphate isomerase (TPI) and *E. bieneusi* and *Encephalitozoon* spp. at partial sequences of gene encoding the ITS of rRNA using established protocols and primers (Didier et al., 1995; Katzwinkel-Wladarsch et al., 1996; Xiao et al., 1999; Buckholt et al., 2002; Sulaiman et al., 2003). PCR amplicons were purified by GenElute™ Gel Extraction Kit (Sigma, USA) according to the manufacturer's instructions and sequenced in both directions (SeqMe, Czech Republic).

Bioinformatics and data assembly of strongylid nematodes

Gene-specific primers were trimmed using *skewer* (Jiang et al., 2014). Next, low-quality reads (expected error rate >2) were eliminated, and the filtered data set was denoised using *dada2* (Callahan et al., 2015) to obtain amplicon sequence variants (ASVs). ASV are unique DNA sequences generated from targeted amplification of specific regions in the genome, allowing for precise identification of distinct taxa. All ASV sequences are deposited in the GenBank (Supplementary Table 1). Naive Bayesian *RDP classifier* (Wang et al., 2007) implemented in the *dada2* pipeline was used for taxonomic assignment of ASVs with 80% posterior confidence. The reference training data set was downloaded from the National Center for Biotechnology Information (NCBI) database (200 top blast hits for each of our ASVs, environmental samples were excluded). To avoid inflation of strongylid diversity due to PCR/sequencing artefacts that were not corrected by *dada2*, we considered only those ASVs that were consistently present in both technical PCR duplicates for a given sample (Pafčo et al., 2018). We also excluded ASVs that did not correspond to the Strongylida order. Read counts for ASVs corresponding to the same genus were grouped within the resulting abundance matrix.

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Phylogenetic analyses for *Strongyloides* and protists

We aligned sequences using Geneious Prime 2024.0.7 (www.geneious.com) and the phylogenetic trees were derived from IQ-tree (Minh et al., 2020) based on the maximum likelihood method with 1000 bootstrap replicates. The sequences from this study are deposited in GenBank (Supplementary Table 2).

Statistics

The prevalence of a parasite taxon was calculated by dividing the total number of individuals with a specific parasite taxon obtained from both microscopic and molecular methods by the total number of individuals of a specific host group sampled, and the parasite species richness as the number of parasite taxa found in each host's faecal samples throughout the study period (Bush et al., 1997). Parasite infection rate was calculated as the presence of at least 1 parasite in the faecal sample divided by the number of samples for a particular host category. Chi-square tests of independence, followed by pairwise comparisons, were conducted to test for significant differences in proportion of samples infected across different host groups as well as proportion of samples infected with each parasite taxa across different host groups.

Strongylid ASV prevalence was defined as the number of hosts infected with each strongylid ASV divided by the number of hosts sampled. Differences in number of reads per host type and relative abundances of strongylid nematode communities among host type were compared using generalized linear models (GLMs) followed by pairwise comparisons. Differences in alpha diversity of strongylid communities were investigated by using the number of ASVs per sample as a proxy measure. The number of different haplotypes per sample was highly correlated with the other commonly used alpha diversity indices, such as Shannon index (cor. coef. = 0.78), Simpson (cor. coef. = 0.63) or inverse Simpson (cor. coef. = 0.78), and thus we used it as the only alpha diversity index in our analyses. The differences in haplotype diversity among host categories were tested by GLM with a quasibinomial error distribution, followed by a Tukey post hoc test for pairwise comparison. Community composition was defined as prevalence and relative representation of ITS2 ASVs using 2 beta diversity metrics: Jaccard and Bray–Curtis ecological distances. Principal coordinate analysis (PCoA) was performed on both Jaccard and Bray–Curtis dissimilarities. Permutational analysis of variance (PERMANOVA) was performed, followed by analysis of similarity (ANOSIM), to test the interspecific differences in strongylid nematode community compositions among the hosts. Multivariate GLMs from the R package mvabund (Wang et al., 2012) were implemented to search for community-wide divergence and identification of significant ASVs that varied due to the different host species effect.

Results

The total number of parasite taxa identified by microscopic and genetic analyses was 33 (Tables 1 and 2, Supplementary Table 3), with dogs harbouring 18 parasite taxa, livestock harbouring 14 parasite taxa, vervet monkeys harbouring 10 parasite taxa and in latrine samples 10 parasite taxa was detected. Using microscopy, cestodes, nematodes and trematodes were observed in 84 samples, whereas all protists were detected exclusively through molecular analyses (Table 1). We further focused on strongylid nematodes and *Strongyloides*. Based on microscopy alone, strongylid nematodes could not be differentiated and could not be reliably assigned to lower taxonomic level; the eggs were detected in 23 samples (dogs: 4, latrines: 1, livestock: 4, vervets: 14). In contrast, HTS enabled the discrimination of individual ASVs (Supplementary Table 1), with 45 samples (dogs: 18, latrines: 12, livestock: 5, vervets: 10) detected positive. Of the 23 samples identified positive by microscopy, only 7 samples were found positive by HTS sequencing. In contrast, HTS sequencing identified 38 positive samples that microscopy had identified as negative. *Strongyloides* eggs were identified in 21 (dogs: 0, latrines: 0, livestock: 1, vervets: 20) samples by microscopy, whereas 33 samples (dogs: 5, latrines: 0, livestock: 1, vervets: 27) tested positive using qPCR. Additionally, 15 samples that tested positive by microscopy were negative by qPCR, while 27 samples positive by qPCR were negative by microscopy.

The microscopic analysis itself showed that the parasitic infection rate was 40% (10/25) in dogs, 24% (6/25) in latrine samples, 44% (11/25) in livestock and 88% (66/75) in vervet monkeys. Dogs (mean ± SE = 0.4 ± 0.1) were found to be more infected than latrine samples (mean ± SE = 0.24 ± 0.09) ($\chi^2 = 5.88$, $df = 1$, $p = 0.02$; Fig. 1) and latrine samples were also less infected than livestock (mean ± SE = 0.44 ± 0.10) ($\chi^2 = 8.91$, $df = 1$, $p < 0.05$; Fig. 1). All hosts were less infected than vervet monkeys (mean ± SE = 0.88 ± 0.04) (latrines: $\chi^2 = 83.12$, $df = 1$, $p < 0.001$, dogs: $\chi^2 = 50$, $df = 1$, $p < 0.001$, livestock: $\chi^2 = 43.14$, $df = 1$, $p < 0.001$; Fig. 1). Other comparisons were not significant.

Strongylid nematodes

In total 1201 808 high-quality ITS2 reads were identified, with an average 22 675.62 reads (SD: 31 227.53; range: 223–166 772) and median sequencing depth of 12 422 reads per sample. In total, 157 individual ASVs were identified, including 7 strongylid genera namely *Ancylostoma, Cooperia, Haemonchus, Hyostrongylus, Murshidia, Oesphagostomum* and *Trichostrongylus* (Table 2). Of the known units, the highest number of ASVs belonged to *Ancylostoma caninum* in dogs, followed by *Haemonchus similis* in livestock (Table 2). In addition, 57 ASVs could not be identified to the species level (present in 16% of the samples), out of which 31 ASVs belonged to genus *Ancylostoma*, 2 ASVs to genus *Cooperia*. 12 ASVs to *Hyostrongylus* and 12 ASVs to order Rhabditida whose further taxonomy could not be assigned, and these are referred to as 'Unassigned Strongyloidea' further along.

The number of reads varied significantly across sample type ($\chi^2 = 15.63$, $df = 3$, $p = 0.001$) (Fig. 2A), with dogs having a higher number of reads compared to latrines ($z = 37845$, SE = 10439, $df = 49$, $t = 3.63$, $p = 0.004$) and vervet monkeys ($z = 27881$, SE = 9337, $df = 49$, $t = 2.98$, $p = 0.02$). The most prevalent variants belonged to 3 genera: *Ancylostoma, Murshidia* and *Oesophagostomum* (Table 2). There was a significant interspecific difference in the relative abundances of strongylid nematode communities among sample type (genus: $\chi^2 = 8.79$, $df = 3$, $p = 0.03$; species: $\chi^2 = 24.24$, $df = 3$, $p < 0.001$) (Fig. 2B). At the genus level, latrine samples harboured fewer genera compared to vervet monkeys ($z = -0.28$, SE = 0.10, $df = 96$, $t = -2.77$, $p = 0.03$; Fig. 2B). At the species level, vervet monkeys harboured more species compared to dogs ($z = -0.37$, SE = 0.08, $df = 124$, $t = -4.85$, $p < 0.001$) and latrine samples ($z = -0.27$, SE = 0.09, $df = 124$, $t = -3.03$, $p = 0.02$; Fig. 2C).

Variant diversity (explored as number of ASVs) differed significantly among sample type (GLM: $F_{(3, 53)} = 21.59$, $p < 0.001$). Variant diversity in vervet monkeys was lower compared to dogs

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Table 1. Prevalence of parasite taxa detected by microscopic and genetic methods and their prevalence in the dogs, latrine samples, livestock and vervet monkeys at the shore of Lake Nabugabo, Uganda

		Sample type			
	Taxa	Dogs ($N = 25$)	Latrines ($N = 25$)	Livestock ($N = 25$)	Vervet monkeys ($N = 75$)
Cestodes	*Spirometra*	8	0	0	0
Nematodes	*Ascaris*	0	4	4	1.3
	Enterobius	0	4	0	0
	Nematodirus	0	0	4	0
	Strongylid eggs	16	4	16	18.7
	Strongylid DNA	72	48	20	13.3
	[a]*Ancylostoma*	56	4	4	0
	[a]*Cooperia*	4	0	0	0
	[a]*Haemonchus*	0	0	4	0
	[a]*Hyostrongylus*	8	12	4	0
	[a]*Murshidia*	24	36	4	0
	[a]*Oesophagostomum*	36	36	16	11
	[a]*Trichostrongylus*	0	0	0	8
	[a]Unclassified Strongyloidea	20	28	0	4
	Strongyloides eggs	0	0	4	26.7
	Strongyloides DNA	20	0	4	36
	[a]*Strongyloides fuelleborni*	0	0	0	36
	[a]*Strongyloides ransomi*	0	0	4	0
	[a]*Strongyloides stercoralis* lineage *A*	8	0	0	0
	Toxocara	8	0	0	0
	Trichuris	0	8	0	78.7
Trematodes	Paramphistomidae	0	0	16	0
Protists	[a]*Cryptosporidium andersoni*	4	0	8	0
	[a]*Cryptosporidium canis*	4	0	0	0
	[a]*Cryptosporidium scrofarum*	0	0	8	0
	[a]*Cryptosporidium ubiquitum*	0	0	8	0
	[a]*Encephalitozoon* spp.	0	0	0	4
	[a]*Enterocytozoon bieneusi*	0	12	0	0
	[a]*Giardia intestinalis*	16	12	0	1.3

[a]Parasite taxa identified from DNA sequencing.

($z = 1.78$, SE $= 0.48$, t ratio $= 3.71$, $p = 0.001$) (Fig. 3), while there was no evidence of significant differences between all other hosts (Fig. 3). PCoA diagrams based on both Jaccard and Bray–Curtis ecological distances confirmed clear differences among studied hosts in both composition and relative abundance of strongylid ASVs (Fig. 4). Significant differences between different sample types in the composition of their strongylid nematode communities were further confirmed by PERMANOVA (Jaccard: $F_{(3,53)} = 1.71, p < 0.001$; Bray–Curtis: $F_{(3,53)} = 2.76, p < 0.001$) and ANOSIM (Jaccard: $R = 0.32$, $p = 0.001$; Bray–Curtis: $R = 0.32$, $p = 0.001$) tests. Tukey post hoc testing revealed significant differences between dogs and latrine samples, dogs and vervets, and latrine samples and vervets for both Jaccard and Bray–Curtis distances ($p < 0.05$), while all other pairwise comparisons were not statistically significant.

Mvabund testing confirmed the interspecific differences (mvabund: $\Delta DF = 3, \chi^2 = 749.1, p < 0.05$) and identified 11 ASVs with whose different relative abundances were the main driving force of diversity among different host species in contrast to shared haplotypes (Supplementary Figure 2). Differences among hosts were mainly due to greater frequencies of *A. caninum, A. duodenale, A. braziliense* in dogs, *Murshidia* sp. in latrine samples and *O. stephanostomum* in vervets (Supplementary Figure 1).

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Table 2. Number of identified amplicon sequence variants (ASVs), their proportion of total reads, number of ASVs in each sample type and ASVs prevalence among host species

			Number of ASVs (Prevalence, %)			
Parasite taxa	Number of identified ASVs	Total reads proportion (%)	Dogs	Latrines	Livestock	Vervet monkeys
Ancylostoma braziliense	16	11.1	16 (32)	0 (0)	0 (0)	0 (0)
Ancylostoma caninum	20	25.3	20 (48)	0 (0)	1 (4)	0 (0)
Ancylostoma duodenale	13	15.3	13 (28)	1 (4)	0 (0)	0 (0)
Ancylostoma tubaeforme	3	5.3	3 (8)	0 (0)	0 (0)	0 (0)
Ancylostoma sp.	31	1.4	31 (28)	0 (0)	0 (0)	0 (0)
Cooperia punctata	3	1.9	3 (4)	0 (0)	0 (0)	0 (0)
Cooperia sp.	2	0.8	2 (4)	0 (0)	0 (0)	0 (0)
Haemonchus similis	18	3.7	0 (0)	0 (0)	18 (4)	0 (0)
Hyostrongylus sp.	12	1.1	2 (8)	3 (12)	12 (4)	0 (0)
*Murshidia*sp.	11	4	10 (24)	11 (36)	1 (4)	0 (0)
Oesophagostomum bifurcum	2	0.5	0 (0)	0 (0)	1 (4)	1 (3)
Oesophagostomum dentatum	4	4.4	2 (4)	0 (0)	4 (4)	0 (0)
Oesophagostomum quadrispinulatum	2	0.2	0 (0)	0 (0)	2 (4)	0 (0)
Oesophagostomum stephanostomum	4	17.7	2 (36)	1 (36)	1 (12)	3 (20)
Trichostrongylus axei	1	0.3	0 (0)	0 (0)	0 (0)	1 (1)
Trichostrongylus colubriformis	3	3.8	0 (0)	0 (0)	0 (0)	3 (8)
Unclassified Strongyloidea	12	3.2	2 (20)	3 (28)	0 (0)	7 (4)

The shaded cells represent the taxa with ASVs detected in multiple sample types.

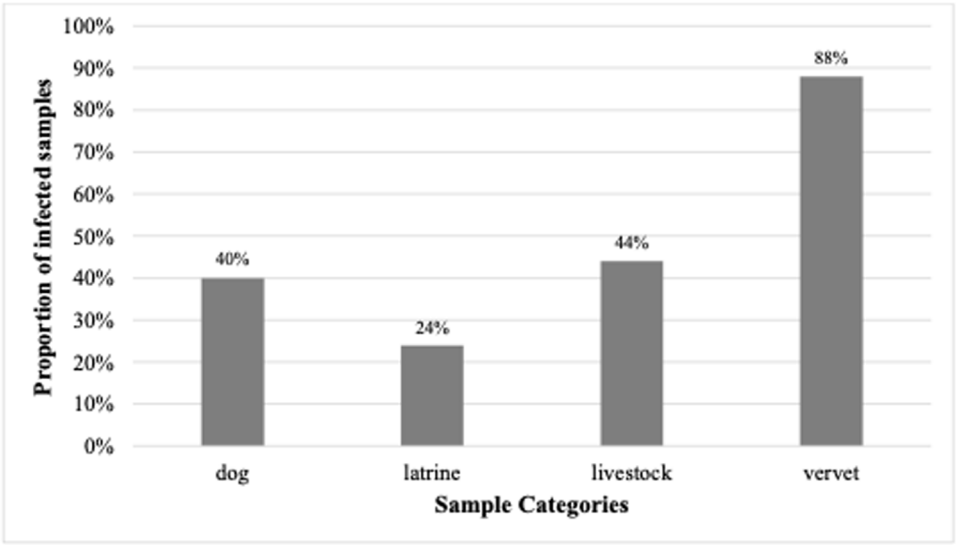


Figure 1. Proportion of infected samples from dogs ($N = 25$), latrines ($N = 25$), livestock ($N = 25$) and vervet monkeys ($N = 75$) at the shore of Lake Nabugabo, Uganda.

Strongyloides

Molecular analysis revealed that 27 vervet monkey samples were infected with *S. fuelleborni*, whose HVR-IV sequences clustered with the isolates on GenBank sampled from non-human primates and humans from East and Central Africa as well as East Asia (Supplementary Figure 3). Dogs were infected with *S. stercoralis* lineage A. This was confirmed by the phylogenetic analysis, as the obtained sequences clustered with *S. stercoralis* lineage A sequences available in GenBank from humans, primates and dogs across Africa, Europe and Southeast Asia (Supplementary Figure 3). Three dogs were also found to be infected with *S. ransomi*, most likely a spurious parasitism as a consequence of eating pig faeces. One pig was also found to be infected with *S. ransomi*. *Strongyloides ransomi* found in dogs and pig clustered with the isolates of *S. ransomi* on GenBank sampled from wild boar in Brazil and the isolates of *S. venezuelensis* from brown rat (*Rattus norvegicus*) in Japan (Supplementary Figure 3).

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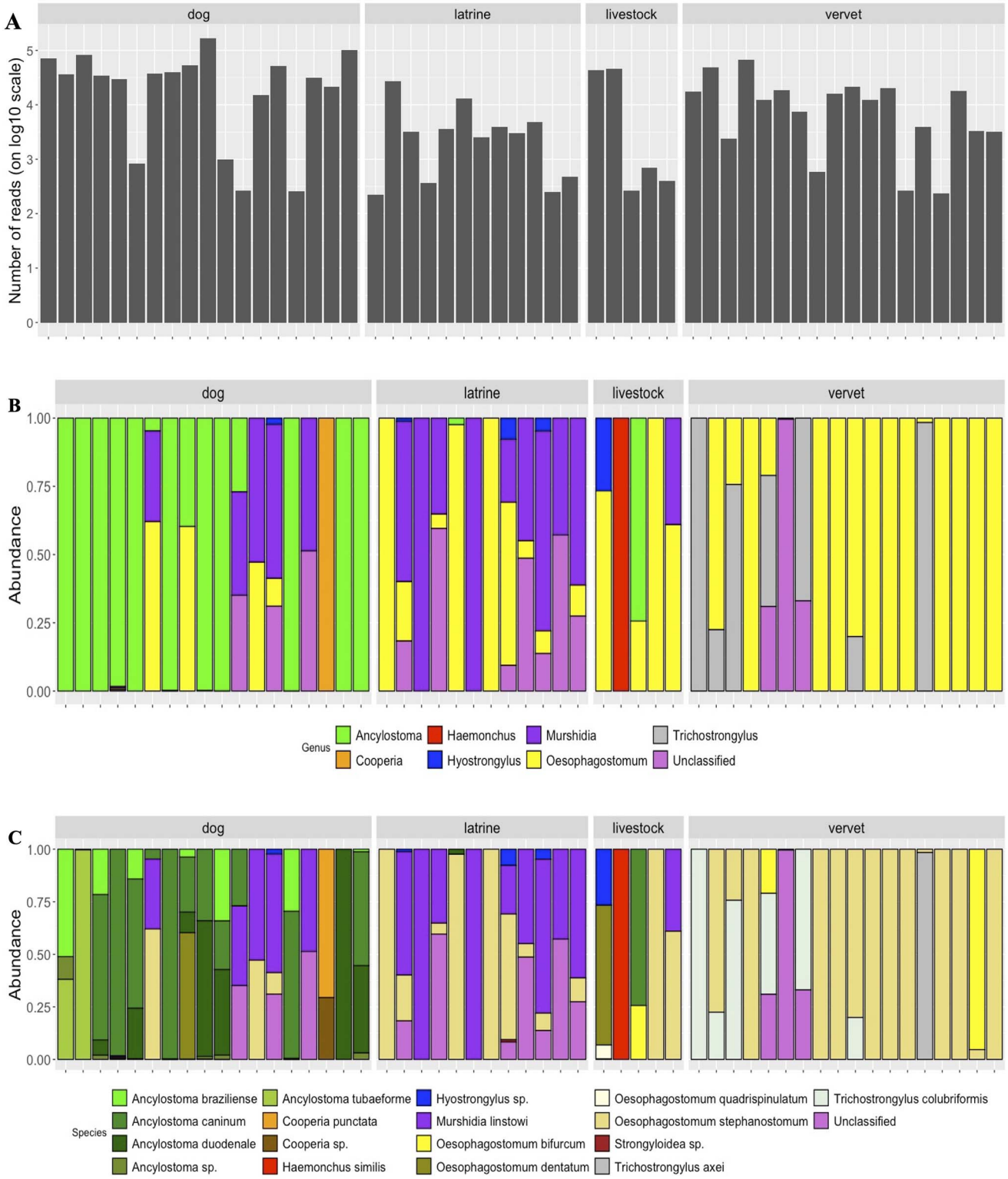


Figure 2. Bar plots showing (A) number of reads in each individual sample on a log10 scale, (B) relative community composition of strongylid nematodes in examined samples at the genus level, (C) relative community composition of strongylid nematodes in examined samples at the species level. Each column represents a sample. Numbers of reads (A)/relative abundances (B, C) of reads are depicted as colour panels.

Protists

Out of the 25 faecal samples collected from livestock, 24% tested positive for *Cryptosporidium* spp., with infection rates ranging from 0 to 8% (Table 1). In dogs (n = 25), 8% were positive, with infection rates from 0 to 4% (Table 1). In this study, 4 *Cryptosporidium* spp., namely *C. andersoni* in 1 dog and 2 cows,

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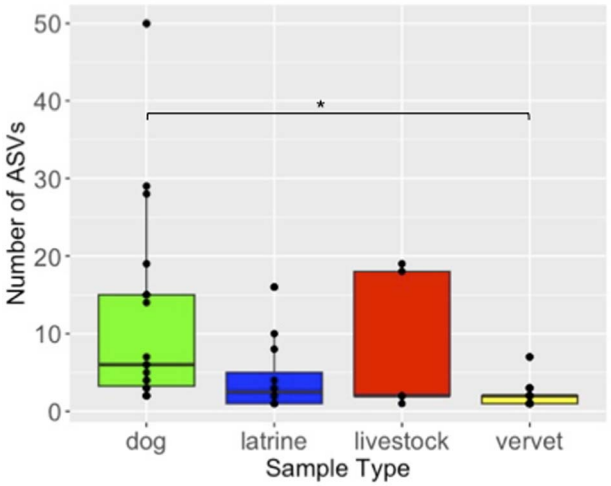


Figure 3. Boxplot of amplicon sequence variants (ASVs) counts according to sample types.
*Significant pairwise comparison.

C. canis in 1 dog, *C. scrofarum* in 2 pigs and *C. ubiquitum* in 2 goats were identified. *Cryptosporidium* 18S rDNA sequences from a dog clustered with the isolates of *C. canis* on GenBank sampled from dogs in Europe and racoon dogs (*Nyctereutes procyonoides*), minks, blue foxes (*Vulpes lagopus*) and dogs in East Asia (Supplementary Figure 4). The *Cryptosporidium* 18S rDNA sequences from a dog also clustered with *C. canis* found in water in Spain (Supplementary Figure 4). The *Cryptosporidium* 18S rDNA sequences obtained from goats clustered with the isolates of *C. ubiquitum* on GenBank sampled from goats and Tibetan sheep (*Ovis ammon hodgsoni*) in China, hosts such as roe deer (*Capreolus capreolus*), common ostrich (*Struthio camelus*), nutria (*Myocastor coypus*) in Europe, alpaca (*Vicugna pacos*) in South America, long-tailed chinchilla (*Chinchilla lanigera*) in East Asia and eastern grey squirrel (*Sciurus carolensis*) and American red squirrel (*Tamiasciurus hudsonicus*) in North America (Supplementary Figure 4).

For *Giardia intestinalis* assemblage A, 16% of dog samples, 12% of latrine samples and 1.3% of vervet monkey samples were positive (Table 1). The TPI sequences obtained from dogs, latrines and vervet monkeys clustered with the isolates of *G. intestinalis* assemblage A on GenBank sampled from rhesus macaque (*Macaca mulatta*), goat, sheep (*Ovis aries*), François' leaf monkey (*Trachypithecus francoisi*), cattle in China, sheep in USA, alpaca in Australia and humans in China, Australia and Malaysia (Supplementary Figure 5).

Enterocytozoon bieneusi was detected in 12% of human household samples (Table 1). The ITS sequences obtained from 2 latrine samples clustered with the isolates of *Enterocytozoon bieneusi* genotype Peru8 on GenBank sampled from human, guereza colobus (*Colobus guereza*), rhesus and Assamese macaques (*Macaca assamensis*) in China and humans in Peru (Supplementary Figure 6). Similarly, the ITS sequence obtained from 1 latrine sample clustered with the isolates of *Enterocytozoon bieneusi* genotype D on GenBank sampled from dogs, golden snub-nosed monkeys (*Rhinopithecus roxellana*) and northern white-cheeked gibbons (*Nomascus leucogenys*) in China, humans in China and Egypt, foxes (*Vulpes vulpes*) in Poland and mountain gorillas (*Gorilla beringei beringei*) in Rwanda (Supplementary Figure 6).

Out of 75 faecal samples collected from vervet monkeys, only 4% tested positive for *Encephalitozoon* species (Table 1). One of the ITS sequences obtained from vervet samples clustered with the isolates of *Encephalitozoon intestinalis* on GenBank from red panda (*Ailurus fulgens*) in China, Central African chimpanzee, moustached guenon monkey (*Cercopithecus cephus*) in Cameroon and humans in Russia and Iran (Supplementary Figure 7). The other 2 ITS sequences obtained from vervet samples were found 86.2% homologous with *Encephalitozoon cuniculi* on GenBank from red panda in China, 83.7% homologous with *E. cuniculi* from mountain gorilla in Rwanda, 82.9% homologous with *E. cuniculi* from cat in Austria and 82.6% homologous with *E. cuniculi* from rabbit (*Oryctolagus cuniculus*) in Japan (Supplementary Figure 7).

Shared parasites

Overall, several parasite taxa were shared among multiple sample types (Tables 1 and 2), indicating potential cross-species transmission or overlapping environmental exposure. Eggs of *Ascaris* sp. were detected across latrine samples, pigs and vervets, whereas eggs of *Trichuris* sp. were detected in latrine samples and vervet monkeys (Table 1). *Hyostrongylus* was present in dogs, latrine samples and a pig, whereas *Murshidia* was shared among dogs, latrine samples and a goat (Table 2). *Oesophagostomum bifurcum* was shared between cattle and vervets, and *O. dentatum* was identified in both a dog and a pig (Table 2). Notably, *O. stephanostomum* was the most widespread, occurring in vervets, pigs, latrine samples and dogs (Table 2). Haplotypes of *O. stephanostomum* were shared across a wide range of hosts, including dogs, latrines, goats, pigs and vervets. *Murshidia* haplotypes were detected in dogs, goats and latrines while *A. caninum* was shared between dogs and cattle. *Ancylostoma duodenale* appeared in both dogs and latrine samples. *Hyostrongylus* haplotypes were found in dogs, latrines and pigs and *O. dentatum* was shared between dogs and pigs (Table 3). *Cryptosporidium andersoni* occurred in dogs and cattle (Table 1). Similarly, *G. intestinalis* was shared among dogs, latrine samples and vervets (Table 1).

Discussion

In this study, parasite prevalence, species richness and infection rate in dogs, latrine samples, livestock and vervet monkeys at Lake Nabugabo, Uganda were assessed by employing both microscopy and molecular techniques, identifying a total of 33 parasite taxa across the 4 sample types. The findings highlight important differences in parasite diversity and prevalence, shedding light on potential parasite transmission dynamics among wildlife, domestic animals and humans, along with species-specific differences in susceptibility or exposure (Fenton and Pedersen, 2005; Bielby et al., 2015).

A higher diversity of parasites was found in dogs, with 18 parasite taxa identified, compared to the other host species, likely due to coprophagy, close contact with humans and exposure to various parasites in domestic environments. Free-roaming dogs at Nabugabo may also be more prone to encountering contaminated areas and shared habitat with wildlife. In general, dogs have been linked to emerging human diseases like eosinophilic enteritis and alveolar echinococcosis and thus may pose risks for people (Salb et al., 2008). Our findings are consistent with established research, which highlights dogs as a major source of zoonotic parasites, facilitating the transmission or transport of these parasites between livestock, wildlife and humans.

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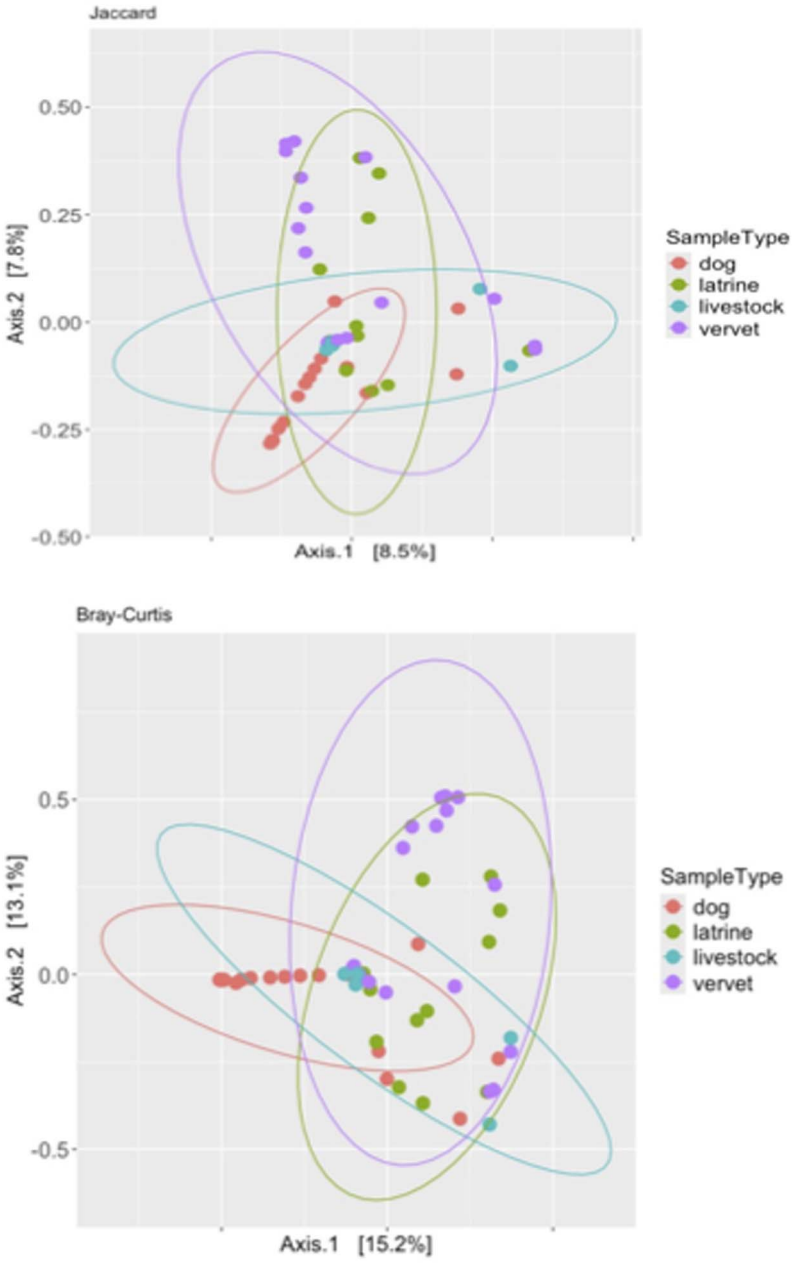


Figure 4. PCoA ordination diagrams of beta diversity of strongylid nematode communities based on Jaccard ecological distance (presence/absence of amplicon sequence variants (ASVs)); Bray–Curtis ecological distance (relative abundances of reads).

Strongylid nematodes

Among the 7 genera of strongylid nematodes found in this study, the most prevalent in dogs was *Ancylostoma*, while it was *Oesophagostomum* in vervets and latrine samples. These strongylids are commonly reported in a wide variety of hosts (Brooker et al., 2004; Ghai et al., 2014; Cibot et al., 2015; Pafčo et al., 2019; Nezami et al., 2023). Interestingly, *Hyostrongylus* and *Murshidia* were also prevalent genera in latrine samples; however, these have never been reported from human samples before.

Ancylostoma caninum, one of the most common parasitic nematodes in dogs globally (Nezami et al., 2023), was found in 12 dogs and in 1 cow, suggesting a possible passage through contaminated soil or water or faeces in that case. *Ancylostoma duodenale* was found in 7 dogs and 1 latrine sample. Although *A. duodenale* was detected in only 1 latrine sample, it is unclear how many individuals used that latrine, meaning that several users could have been infected and the true prevalence in the local population may be higher. Other studies have reported high prevalence of this parasite in humans, clearly confirming humans as its host (Adenusi and Ogunyomi, 2003; Pafčo et al., 2019). One previous study documented its presence in humans in the Dzanga-Sangha Protected Areas of the Central African Republic while the human samples were dominantly infected by *Necator* (Pafčo et al., 2019). Although *A. duodenale* often coexists with *Necator americanus* in many endemic areas (Adenusi and Ogunyomi, 2003), we did not detect any *Necator* sequences in this study. *Ancylostoma duodenale* is considered a human-specific hookworm, with humans recognized as the only natural definitive host (CDC, 2024b) and its detection in the canine samples is likely a result of coprophagy of human faeces. Nevertheless, dogs may represent an

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Table 3. Amplicon sequences variants (ASVs) shared among different sample types

ASV	Genus	Species	Sample type
ASV10	*Ancylostoma*	*Ancylostoma caninum*	Dog, cattle
ASV12	*Ancylostoma*	*Ancylostoma duodenale*	Dog, household latrines
ASV18	*Hyostrongylus*	*Hyostrongylus* sp.	Dog, household latrines, pig
ASV23	*Hyostrongylus*	*Hyostrongylus* sp.	Household latrines, pig
ASV34	*Hyostrongylus*	*Hyostrongylus* sp.	Dog, household latrines, pig
ASV3	*Murshidia*	*Murshidia* sp.	Dog, household latrines
ASV5	*Murshidia*	*Murshidia* sp.	Dog, household latrines, goat
ASV6	*Murshidia*	*Murshidia* sp.	Dog, household latrines
ASV7	*Murshidia*	*Murshidia* sp.	Dog, household latrines
ASV16	*Murshidia*	*Murshidia* sp.	Dog, household latrines
ASV28	*Murshidia*	*Murshidia* sp.	Dog, household latrines
ASV44	*Murshidia*	*Murshidia* sp.	Dog, household latrines
ASV129	*Murshidia*	*Murshidia* sp.	Dog, household latrines
ASV2	*Oesphagostomum*	*Oesophagostomum stephanostomum*	Dog, household latrines, goat, pig, vervet
ASV19	*Oesophagostomum*	*Oesophagostomum dentatum*	Dog, pig
ASV4	Unassigned	Unclassified Strongyloidea	Dog, household latrines
ASV8	Unassigned	Unclassified Strongyloidea	Dog, household latrines

important reservoir contributing to parasite transmission, as has been previously demonstrated for *N. americanus* (Boyko et al., 2020).

Dogs in this study were also infected with 3 additional species of *Ancylostoma: A. braziliense, A. tubaeforme* and an unclassified *Ancylostoma* species. While *A. tubaeforme* is typically considered a feline parasite, it is genetically and biologically related to dog hookworms such as *A. caninum* and *A. braziliense*. Notably, *A. braziliense* has been previously reported in cats in the United States of America, demonstrating its ability to infect multiple host species (Liotta et al., 2012). Therefore, although presence of the feline species *A. tubaeforme* in dogs could be a consequence of coprophagy, it may also be a case of potential cross-species transmission. These findings underscore the diversity of hookworm infections in dogs and the potential for interspecies overlap among *Ancylostoma* species. Moreover, presence of these *Ancylostoma* species represents a risk for public health as canine hookworms cause larva migrans cutanea in humans (Nezami et al., 2023).

Murshidia was found in dogs, latrine samples and livestock (goats). *Murshidia* species are primarily known to infect elephants (*Loxodonta* spp.), warthogs and other herbivores (Chel et al., 2020; Malviya et al., 2024). To our knowledge, this is the first report of *Murshidia* in goats and dogs, although in the latter case is most likely a spurious parasite. The detection of *Murshidia* in human samples requires further consideration. It is possible that the presence of the parasite in human latrine samples was influenced by the method of sample collection. Specifically, the samples were obtained from pit latrines, where various materials, including livestock faeces, could have been discarded. This creates a risk of cross-contamination, which might explain the unexpected finding of *Murshidia* in human samples. It is also important to note that multiple individuals may have used the same pit latrine, and/or a single individual may have used more than 1 pit latrine, potentially resulting in multiple 'pit latrine' infections.

This study identified 4 species of *Oesophagostomum* across different host species, highlighting the broad distribution of these parasites in both domestic and wild animals, as well as humans. A single pig sample was found to be infected with *O. stephanostomum, O. quadrispinulatum* and *O. dentatum*. Similar taxa were reported in Tibetan pigs (Li et al., 2017), although the identification of *O. stephanostomum* in that study remains uncertain due to the low BLAST sequence identity and incongruent phylogenetic placement relative to authentic *O. stephanostomum* references. Given that pigs are not typical hosts for *O. stephanostomum*, its occurrence here could represent a spurious infection or an unrecognized cross-host transmission event, both of which warrant further molecular investigation.

Two *Oesophagostomum* species are commonly found in both human and non-human primates in Africa: *O. stephanostomum* mostly in great apes and occasionally in humans (Cibot et al., 2015; Narat et al., 2015; Ilík et al., 2023) and *O. bifurcum* in humans and various monkeys (Ziem et al., 2006; Ghai et al., 2014; Sirima et al., 2021). We detected both species in vervet monkeys with *O. stephanostomum* having higher prevalence and this species was also found in human latrine samples. While there is not much information available on pathogenicity of *O. stephanostomum* in humans, *O. bifurcum* is known cause of potentially severe illness in some areas of West Africa (Ziem et al., 2006). Their presence in the area deservers further attention, which should focus on a detailed epidemiology and clinical picture of the infection in humans. The finding that *O. dentatum* was shared between pigs and dogs is consistent with previous studies suggesting that *Oesophagostomum* species can be maintained in multiple definitive host species (Ghai et al., 2014; Cháves-González et al., 2022), further complicating the ecological dynamics of these parasites; that said, the presence of *O. dentatum* in dogs is more likely a result of coprophagy.

Among other strongylid nematodes detected in various hosts, genus *Hyostrongylus* was found in human, dog and pig samples. *Hyostrongylus* is typically found in pigs (Kouam and Ngueguim, 2022), and the presence of *Hyostrongylus* in dogs could be a spurious infection, while its presence in humans could be a result of sampling method from pit latrines. Thus, this finding requires further examination. In this study, variants of *Cooperia punctata* and a *Cooperia* sp. were found in dogs, which could be the result of coprophagy. The genus *Cooperia* belongs to Trichostrongyloid nematodes that are important in veterinary medicine, as they play a role in mixed-species helminth infections, leading to production losses in livestock worldwide (Stromberg et al., 2012).

Dogs exhibited higher strongylid alpha diversity than vervets. This high diversity among dogs was attributed primarily to the

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prevalence and dominance of *A. caninum* variants, which not only exhibited high infection rates but also contributed substantially to the overall relative abundance of strongylid nematodes in the host population. Specifically, dogs were found to harbour 11 distinct species of strongyle nematodes, raising the possibility of dogs as a major reservoir of these parasites. However, it should be highlighted that most of these infections are likely due to coprophagy of infected faeces from animals or humans. That said, dogs can still spread eggs or larvae that may remain infectious to other hosts (e.g. in case of *N. americanus* (Boyko et al., 2020)) which warrants further investigation. On the other hand, the strongylid communities of humans, livestock and vervets were dominated by variants belonging to *O. stephanostomum, T. colubriformis* and *Murshidia* sp. As previously noted, however, the detection of *Murshidia* sp. in human samples warrants careful consideration.

Strongylid nematodes are capable of surviving in their hosts for years and infection typically do not lead to death (Ilík et al., 2023). In fact, none of the sampled animals displayed obvious symptoms of severe infections such as respiratory difficulties, anaemia, weight loss, diarrhoea, worsened quality of hair, apathy, inappetence, etc. (Ilík et al., 2023). Dogs, which showed the highest strongylid diversity in this study, could, therefore, contribute to environmental contamination even when infections are subclinical. Similarly, vervets and livestock, especially goats and pigs, harboured several taxa usually linked to other host species, suggesting they may sustain these parasites without noticeable pathology. Such asymptomatic infections can support cross-species transmission and complicate our understanding of local parasite dynamics and zoonotic risk. Moreover, even if the infection will not establish and the parasites stages just pass through the digestive tract of the host, these individuals then serve as vectors and may spread the stages in the environment (Boyko et al., 2020).

Strongyloides

Despite identifying *S. fuelleborni* in vervet monkeys and *S. stercoralis* in dogs, both of which are *Strongyloides* species that have been previously found in humans (Hasegawa et al., 2009, 2016b; Schär et al., 2014; Thanchomnang et al., 2017), we did not detect any *Strongyloides* infections in human latrine samples at Lake Nabugabo, Uganda. Of particular concern is *S. stercoralis*, a soil-transmitted helminth known to infect humans, non-human primates and dogs (Olsen et al., 2009). Globally, *S. stercoralis* represents a significant public health concern because it can establish lifelong infections through autoinfection, often remaining undetected for decades, and can progress to hyperinfection or disseminated strongyloidiasis in immunocompromised individuals (Keiser and Nutman, 2004; Buonfrate et al., 2013). In humans, clinical manifestations range from mild or asymptomatic infection to gastrointestinal, respiratory and dermatological symptoms and in cases of hyperinfection, patients may develop severe disseminated disease and sepsis, carrying more than 60% mortality rates (Buonfrate et al., 2013; Geri et al., 2015). Some estimates suggested that *S. stercoralis* infects over 600 million people and is the main species responsible for strongyloidiasis (Buonfrate et al., 2020). Although widespread community outbreaks are uncommon, *S. stercoralis* clusters have been documented in institutional settings, including long-term facilities, mental health institutions and among populations with poor sanitation (Schär et al., 2013).

While human infections with *S. fuelleborni* have also been reported, these are rare and are typically associated with individuals who frequent areas inhabited by non-human primates, where *S. fuelleborni* is believed to originate (Hasegawa et al., 2010). Nevertheless, there are rare cases of imported and asymptomatic *S. fuelleborni* infection in people. For instance, a Belgian student who returned from the Democratic Republic of the Congo was treated at the Institute of Tropical Medicine in Antwerp for fever, rash and diarrhoea, where stool microscopy revealed *Trichuris trichiura* and *Strongyloides* eggs, leading to a molecularly confirmed diagnosis of *S. fuelleborni* infection (Potters et al., 2020). Although we did not detect human infections in this study, the presence of this species in vervet monkeys and dogs indicates a potential risk of zoonotic transmission, which has been confirmed in humans, dogs and gorillas in the Central African Republic (Nosková et al., 2026). This is especially concerning given the close proximity between humans and these animals, as well as the possibility of human exposure to environmental contamination with parasite larvae. This makes *S. stercoralis* a major cause of long-term health issues and suffering (Vadlamudi et al., 2006; Prendki et al., 2011; Schär et al., 2013). A pig sample and 3 dog samples were found to be infected with *S. ransomi. Strongyloides ransomi* is known to infect pigs globally (Giang et al., 2017); thus, finding pig and dog samples infected with *S. ransomi* was not surprising. However, dogs might have been positive because of consumption of pig faeces, resulting in spurious parasitism.

Protists

Cryptosporidium, Giardia. and microsporidia species are capable of infecting a broad range of hosts and crossing species barriers, owing to their low host specificity (Thompson, 2004; Franzen, 2008). Despite numerous reports of *Cryptosporidium* spp. in primates (Lim et al., 2008; Karim et al., 2014; Sak et al., 2014; Butel et al., 2015; Mynářová et al., 2016), our study did not detect any *Cryptosporidium* infections in either latrine samples or vervet monkeys. *Cryptosporidium andersoni*, a common species in ruminants (Lindsay et al., 2000; Kváč et al., 2006), was detected only in 2 cows in this study. The finding that only a small number of host samples tested positive for other *Cryptosporidium* species – such as *C. andersoni* and *C. canis* in 2 dogs, *C. scrofarum* in 2 pigs and *C. ubiquitum* in 2 goats suggests that these species may be present at a low prevalence in the studied populations. Although the prevalence across all host species was low, the presence of these *Cryptosporidium* species remains epidemiologically relevant. As many domestic animals can shed oocysts even in the absence of clinical symptoms, the infected cows, dogs, pigs and goats identified here could act as asymptomatic reservoirs (Hatam-Nahavandi et al., 2019). This has potential implications for environmental contamination, particularly in shared water sources or areas where humans and wildlife overlap (Berhanu et al., 2022). At Nabugabo, cows are often brought to the lake to drink where they likely defecate in water that is sometimes used for laundry or swimming. Thus, even low-level circulation of these species may contribute to transmission risk for more vulnerable hosts, including young animals, immunocompromised individuals and wildlife species with higher susceptibility (Berhanu et al., 2022).

Among the 2 assemblages (A and B) of *G. intestinalis* most commonly reported in humans, dogs, cats, livestock and wild animals including non-human primates (Feng and Xiao, 2011; Cama and Mathison, 2015; Kváč et al., 2017), our study identified assemblage A in latrine samples, dogs and a vervet monkey, suggesting a potential for zoonotic transmission. *Giardia*, primarily assemblages A and B, is among the most prevalent intestinal parasites affecting humans, with an estimated 200 million people in Asia, Africa and Latin America suffering from symptomatic infections (Yason and Rivera, 2007). The presence of assemblage A in both

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latrine samples and animals at Lake Nabugabo underscores the potential role of animals as reservoirs for human infections, particularly given that there is habitat overlap between human populations, domestic animals and vervet monkeys.

In this study, *E. bieneusi* was detected exclusively in latrine samples, which aligns with previous research indicating that this species is the primary causative agent of microsporidiosis in humans. *Enterocytozoon bieneusi* is responsible for over 90% of human microsporidiosis cases worldwide, making it the most significant species of microsporidia in terms of public health impact (Xu et al., 2016; Khanduja et al., 2017; Qiu et al., 2019; Tao et al., 2020). However, one surprising and somewhat unexpected finding of this study is that only 12% of latrine samples tested positive for *E. bieneusi*. This low prevalence within latrine samples contrasts with the global distribution of *E. bieneusi* infections and raises important questions about the factors influencing the parasite's transmission in this region. Given the established zoonotic potential of *E. bieneusi* and its known presence in a variety of hosts, including livestock, pets and wildlife (Tao et al., 2020), one might expect a higher prevalence of infection within households where humans often interact with animals. One possibility is that household-level transmission may be less common in this region, either due to effective sanitation practices or the fact that *E. bieneusi* may primarily be transmitted through waterborne routes, as is commonly seen in other endemic areas (Xu et al., 2016); while humans sometimes use lake water for laundry and swimming, well-water is available. In humans, *E. bieneusi* infections are often asymptomatic or mild, especially in immunocompetent individuals, but when symptoms occur, they typically include watery diarrhoea, abdominal discomfort and malabsorption (Nourrisson et al., 2024). From a veterinary perspective, *E. bieneusi* is also important because it infects a wide range of domestic and wild animals, and these animals can act as reservoirs for human infection (Taghipour et al., 2022). Another potential explanation for the low prevalence of *E. bieneusi* in household samples could be related to the diagnostic methods employed in this study. While molecular techniques such as PCR are highly sensitive, the detection of *E. bieneusi* can sometimes be limited by the timing of sample collection or the presence of low parasite loads. Additionally, because of the use of pooled pit latrine samples, it is not possible to determine the exact number of infected people or track individual infection dynamics. Therefore, it is possible that the 12% prevalence reflects only the most overt cases of infection, while subclinical or lower-level infections may have gone undetected.

Finally, a microsporidium closely related to *Encephalitozoon intestinalis* was detected only in 4% ($N = 3$) of vervet monkey samples. Although we did not detect this microsporidia or *E. intestinalis* in other host species, including human latrine samples, previous research has shown that *E. intestinalis* does infect humans in other regions. Studies have reported *E. intestinalis* infections in humans, particularly in immunocompromised individuals, such as those with HIV/AIDS or undergoing chemotherapy (Sokolova et al., 2011; Tavalla et al., 2017). The low prevalence of *E. intestinalis* observed in vervet monkeys in this study may reflect similarly low prevalence rates in other animal species in the region, which could be influenced by factors such as local environmental conditions and hygiene practices.

Limitations

This study has a few important limitations. Because we relied on pooled pit latrine samples, we could not link parasite detections to specific individuals, and heavily infected people may have contributed disproportionately to what we found. Some degree of cross-contamination within latrines is also possible. Pit latrines are common areas where human waste is discarded, but they can also contain other materials, including livestock and dog faeces. The mixing of human and animal waste in these environments could raise the risk of cross-contamination, particularly with parasites that rely on livestock or dogs as their definitive and/or reservoir hosts, which may compromise the accuracy of the findings. Another limitation of our study is that our molecular approach detects parasite DNA but cannot tell whether it reflects an active infection or simply the transient passage of ingested material, which is especially relevant for unexpected host–parasite matches. Furthermore, our sampling was cross-sectional, it captures only a single point in time and does not allow us to determine transmission direction or seasonal patterns. Additionally, we were not able to sample all potential hosts or environmental sources, such as soil or water, which limits how fully we can describe the transmission system. Finally, discrepancies between microscopy and molecular detection were observed. DNA-positive microscopy-negative cases are consistent with the higher sensitivity of molecular methods, which can detect low-intensity infections that may be missed under the microscope. In contrast, microscopy-positive DNA-negative results may stem from occasional difficulties in distinguishing similar morphological features or from the uneven distribution of parasites stages within faecal samples. These differences reflect the inherent strengths and limitations of each diagnostic method and should be taken into account when interpreting the findings. Future work with longitudinal sampling, more detailed genotyping and environmental monitoring will be important for resolving transmission pathways and better understanding the associated risks.

Conclusion

This study highlights the diverse and complex patterns of parasitic infections found in humans, domestic animals and wildlife, with dogs and livestock showing the highest diversity, and vervet monkeys and livestock showing the highest infection rate. The 33 parasite taxa identified across different sample types emphasize the ecological connections among the host species and the potential zoonotic risks involved. Our findings carry important implications for both public health and local livelihoods. Several parasites detected can cause disease even in healthy individuals, highlighting real risks for nearby communities. Livestock parasites like *Trichostrongylus* and *Haemonchus* also suggest potential production losses in goats and cattle, which can impact households that depend on small-scale farming. Because humans, livestock, dogs and vervet monkeys live in close contact around Lake Nabugabo, this area functions as a shared transmission hotspot where zoonotic and anthroponotic parasites can circulate easily. Although we did not formally assess parasite-prevention practices, several local factors likely affect transmission. Dogs roam freely, and most livestock, except pigs, graze in open areas, increasing environmental contamination. Water is mainly drawn from wells (*shaduf*) or the lake. Some residents boil water or wear footwear, but it is unclear how consistently these practices are followed or whether they are used specifically for parasite prevention. These conditions suggest that exposure to contaminated soil and water is common and may help explain some of our results.

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https://doi.org/10.1017/S0031182026101978

Supplementary material. The supplementary material for this article can be found at https://figshare.com/s/56daa2a3c059b68ae1d8.

Data availability statement. Data are available at: https://figshare.com/s/7183b93db8e369344c6a.

Acknowledgements. We thank the Uganda Wildlife Authority (UWA), the Uganda National Council for Science and Technology (UNCST), the Makerere University School of Health Sciences Research Ethics Committee (MAKSHSREC) and the School of Veterinary Medicine and Animal Resources Institutional Animal Care and Use Committee (SVAR IACUC) for granting permission to conduct this research. We thank Dr Dennis Twinomugisha, Matovu Ponsyano, Katwere Livingstone and Justine Namuyomba for logistical support and assistance with the data collection. We also extend our sincere appreciation to the residents of Bbaale village for their willingness to participate and provide samples. Finally, we thank Lordrick Alinaitwe and Ester Nansubuga at Makerere University, Uganda for conducting the microscopic analysis of the samples. OpenAI GPT-5 (i.e. ChatGPT) was used for light editing (e.g. grammar verification).

Author contributions. PU conceived and designed the study, collected the samples and conducted statistical analyses. BC, MK, KS, BP, EN, VI and NH conducted genetic analyses. PU, BC, MK, KS, BP, EN, VI and VAMS wrote and edited the manuscript. PU and VAMS acquired the funding.

Financial support. Funding for the research was provided by the Anne Innis Dagg Grant from the Canadian Society of Zoologists (PU), NSERC and New Frontiers in Research Fund (VAMS). The work of BC and BP was funded by the Czech Science Foundation (22-16475S).

Competing interests. The authors declare there are no conflicts of interest.

Ethical standards. Research was approved by York University (ACC-2016-7W, ACC-2020-06; HPRC-E2019-191), Uganda Wildlife Authority (UWA EC 361 and COD-96-05), Uganda National Council for Science and Technology (NS349ES) and School of Veterinary Medicine and Animal Resources, Institutional Animal Care and Use Committee (SVAR_IACUC/125-2022).

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Barelli, C., Pafčo, B., Manica, M., Rovero, F., Rosà, R., Modrý, D., Haufe, H.C.

2020

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Sci. Rep. 10, 10917. https://doi.org/10.1038/s41598-020-67959-7

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Loss of protozoan and metazoan intestinal symbiont biodiversity in wild primates living in unprotected forests

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In light of the current biodiversity crisis, investigating the human impact on non-human primate gut biology is important to understanding the ecological significance of gut community dynamics across changing habitats and its role in conservation. Using traditional coproscopic parasitological techniques, we compared the gastrointestinal protozoan and metazoan symbiont richness of two primates: the Udzungwa red colobus (*Procolobus gordonorum*) and the yellow baboon (*Papio cynocephalus*). These species live sympatrically in both protected and unprotected forests within the Udzungwa Mountains of Tanzania with distinct ecological adaptations and diets. Our results showed that terrestrial and omnivorous yellow baboons had 2 (95% CI 1.47–2.73) and 3.78 (2.62–5.46) times higher gut symbiont richness (both including and excluding rare protozoans) compared to the arboreal and leaf-eating Udzungwa red colobus in unprotected and protected forest, respectively. We also found a consistent depletion of symbiont richness in red colobus living in the unprotected forest fragment compared to the continuous protected forests [the latter having 1.97 times (95% CI 1.33–2.92) higher richness], but not in yellow baboons. Richness reduction was particularly evident in the Udzungwa red colobus monkeys, confirming the pattern we reported previously for gut bacterial communities. This study demonstrates the impact of human activities even on the microbiodiversity of the intestinal tract of this species. Against the background of rapid global change and habitat degradation, and given the health benefits of intact gut communities, the decrease in natural gut symbionts reported here is worrying. Further study of these communities should form an essential part of the conservation framework.

Human exploitation of natural resources and habitats has caused devastating loss of biodiversity, including both macro-[1–3] and microorganisms[4–6]. Given their critical role in host health and nutrition, the role of bacterial communities, especially the gut microflora, have been a particular focus of attention in recent years, even in wildlife and within a conservation framework[7–9]. However, other relevant components inhabiting the host gastrointestinal tract, such as protozoans and metazoans [hereafter 'gut symbionts', since all symbiotic relationships (from mutualism to commensalism to parasitism[10,11]) are possible], did not receive similar attention. Although traditionally considered harmful to the hosts[12], they are now recognized essential player (i.e. as selective agents[13,14] and modulators of host behavior[15,16]) and potentially beneficial to host health (i.e. regulating gut homeostasis by

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restoring bacterial diversity[17]). Thus, ignoring the conservation of this gut component means neglecting a whole set of biological relationships essential for survival, since host–gut symbiont interactions are among the more prevalent ecological and evolutionary drivers of biological diversity and ecosystem composition[18,19]. Several authors have suggested that such gut symbionts should be recognized as meaningful conservation targets along with their hosts[20,21].

As a result of habitat degradation and/or contraction, studies have revealed that distribution and population size of most animal hosts decreases. If we assume that environmental changes due to human impact similarly affect gut symbionts, we would expect a decrease in prevalence and richness of gut symbionts as well[22–25]. Thus, comparing gut symbiont communities in threatened animal hosts, such as non-human primates[26] living in both protected and human-impacted habitats, is of particular relevance for understanding the impact of conservation efforts and whole ecosystem health[27].

The Udzungwa Mountains of Tanzania (hereafter referred as the Udzungwas) are one of 35 internationally-recognized biodiversity hotspots (https://www.conservation.org)[28]; however, intensive agriculture is currently encroaching on these relatively intact forests[29], making non-human primates living outside protected areas particularly vulnerable[30,31]. We recently discovered that gut metazoan richness of the endemic Udzungwa red colobus monkey (*Procolobus gordonorum*) varies with altitude, with lower richness at lower altitudes[32]. Because human activities are concentrated at lower altitudes, we suggested that anthropogenic disturbance might play an active role in reducing gut symbiont presence and transmission. Therefore, in the present paper, we investigated gut symbiont richness of two sympatric primate species with contrasting life history traits. The endangered Udzungwa red colobus are relatively small canopy dwellers that feed predominantly on leaves[33]; instead, the larger yellow baboons (*Papio cynocephalus*) of least conservation concern, live primarily on the ground and are generalist feeders, with diets including seeds, ripe fruits and animal prey. They are also frequent raiders of human food (i.e. crops and organic waste in villages[34]). Because gut symbiont transmission are likely to depend on host habits and lifestyle, we compared the richness of gastrointestinal protozoans and metazoans of two cercopithecines with highly different ecological adaptations but living sympatrically in contrasting habitat type, predicting higher gut symbiont richness in terrestrial hosts rather than arboreal ones. Moreover, within host species, we predict gut symbiont richness to be higher in hosts living in protected rather than unprotected forest.

Results

Gut symbionts found in the 167 analyzed fecal samples were categorized into one of 10 taxa (five for protozoans and five for metazoans). Optical microscopy did not always allow for species-level identification of symbiont eggs and cysts; thus, gut symbionts were classified to species (*Entamoeba coli*, *Iodamoeba buetchlii*), genus (*Entamoeba* sp., *Blastocystis* sp., *Balantioides* sp., *Trichuris* sp., *Strongyloides* sp.) or higher taxonomic level (strongylids, spirurids, dicrocoeliid trematodes; Fig. 1). Five of these taxa were observed in both primate species (*Strongyloides* sp., *Trichuris* sp., spirurids, strongylids, and *Balantioides* sp.) with prevalence of each taxa ranging between 2 and 90% (Fig. 2, SI Table S1). The remaining five were present only in baboons (Fig. 2). The majority of samples analyzed (80.2%) contained at least one symbiont. More specifically, all baboon samples but one were infected (98.5%; 68/69), while 67.3% (66/98) of red colobus samples resulted infected (Fig. 3). No metazoan larvae or adults were detected with the gauze-washing method.

Gut symbiont richness across primate species. In both protected Mwanihana (MW) and unprotected Magombera (MA) forests, yellow baboons had higher mean symbiont richness compared to Udzungwa red colobus, both including and excluding rare protozoans (GLM: Z value: 7.081, df = 136, p value < 0.001; Fig. 3, Table 1; SI Fig. S1, SI Table S2). Based on Binomial GLM analysis, the prevalence of *Strongyloides* sp., strongylid and spirurid eggs, as well as cysts of *Balantioides* sp. were all significantly higher in baboons than in red colobus as reported in Table 2. Instead, *Trichuris* sp. was found in a higher proportion of red colobus samples compared to those of baboons (Binomial GLM: Z value = -1.992, df = 163, p value = 0.0463; Table 2). Shedding intensities revealed that baboons had higher mean eggs/cysts per gram than red colobus (SI Table S3) for both *Strongyloides* sp. (yellow baboons: eggs' median 434.2, interquantile range 69.1–1,125; red colobus: eggs' median 63.8, interquantile range 35.9–170; p value = 4.18, df = 100, p value = < 0.0001) and *Balantioides* sp. (yellow baboons: cysts' median 762.4, interquantile range 193.7–4,232.9; red colobus: cysts' median 28.2, interquantile range 22.6–44.4; T value = 6.46, df = 72, p value = < 0.0001).

Impact of habitat on gut symbiont richness. Results from the Poisson GLM (Table 1) suggest that yellow baboons inhabiting MA had 3.78 [95% confidence interval (CI) 2.62–5.46] times more symbionts than Udzungwa red colobus monkeys sharing the same habitat type; while in MW, yellow baboons had about 2 (95% CI 1.47–2.7) times more gut symbionts than red colobus. Moreover, gut symbiont richness of red colobus was on average 1.97 times (95% CI 1.33–2.92) higher in individuals living in MW than in those inhabiting MA. In contrast, the same pattern was not observed in yellow baboons where the difference in symbiont richness between forests was not statistically significant (p value > 0.05; Fig. 4, SI Fig. S2). Gut symbiont distribution and prevalence varied with the taxon investigated. For example, in red colobus, a higher prevalence of *Strongyloides* sp. and *Trichuris* sp. were observed for individuals living in MW compared to MA, but not for the other three taxa (Fig. 2, Table 2; SI Table S1). In baboons, only a higher prevalence of *Trichuris* sp. (Fig. 2, Table 2; SI Table S1) was reported in MW compared to MA. Moreover, from the linear model (SI Table S3), egg shedding intensity of *Strongyloides* sp. resulted to be higher in red colobus in MA (median 85.7, interquantile range 48.6–513.4) than in MW (median 50, interquantile range 31.3–146.4). This trend was not observed in baboons (MA: median 442.6, interquantile range 61.9–1,029.8; MW: median 280.6, interquantile range 86.3–1,357.1).

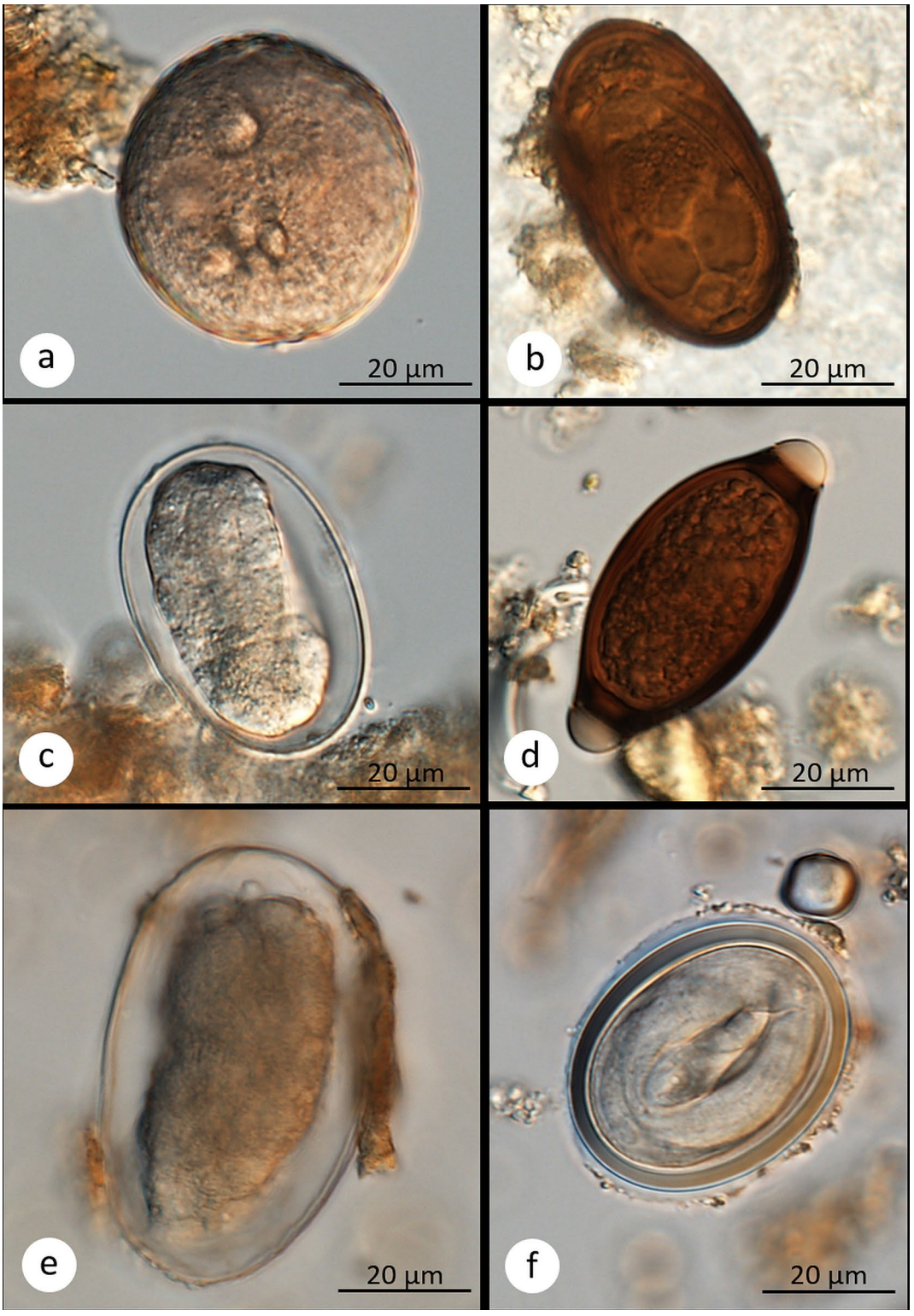


Figure 1. Digital microscopic images of six gut symbionts found in the fecal pellets of yellow baboons and Udzungwa red colobus monkeys from the Udzungwa Mountains of Tanzania: Protozoan: (**a**) cyst of *Balantioides* sp., and eggs of the following metazoans: (**b**) a dicrocoeliid trematode, (**c**) *Strongyloides* sp., (**d**) *Trichuris* sp., (**e**) a strongylid nematode, (**f**) a spirurid nematode.

Finally, modeling showed that overall, altitude influenced neither gut symbiont prevalence nor richness. Only the egg shedding intensity of *Strongyloides* sp. appeared to be influenced by this parameter (SI Table S4).

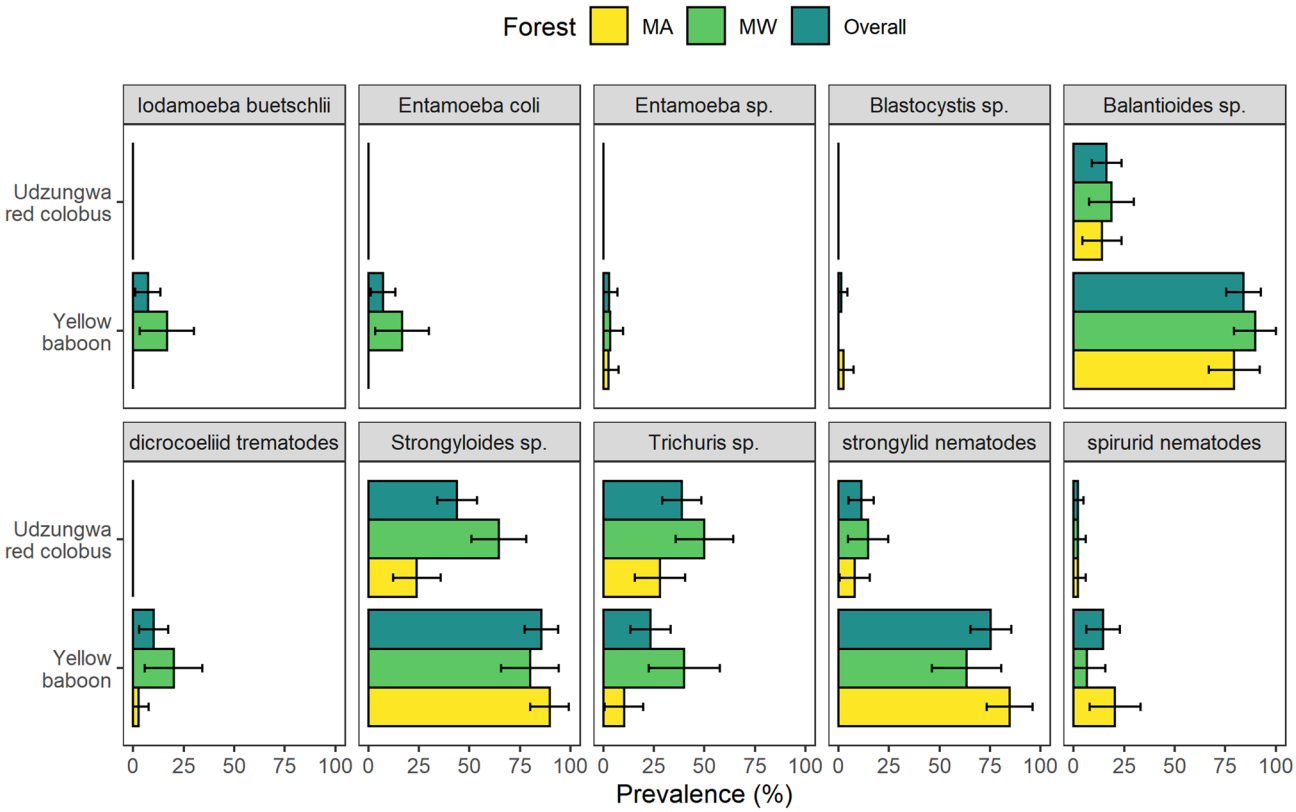


Figure 2. Mean prevalence (expressed in %) of intestinal protozoan and metazoan symbionts in two primate species: Udzungwa red colobus (*Procolobus gordonorum*) and yellow baboon (*Papio cynocephalus*). Colored bars represent the mean symbiont prevalence found from fecal samples of red colobus (N = 69) and yellow baboons (N = 69) collected in two forest types within the Udzungwa Mountains of Tanzania: the unprotected Magombera (MA, colored in yellow) and the protected Mwanihana (MW, light green) forests. Dark green bars indicate the overall mean prevalence for both forest types. Horizontal bars represent the 95% confidence interval.

Discussion

In this study, we investigated whether habitat degradation impact on protozoan and metazoan richness and prevalence of two wild primate species having different ecological adaptations (terrestrial or arboreal), dietary strategies (omnivorous or folivorous) and living in protected and unprotected forests within an Afrotropical biodiversity hotspot. Through the analysis of non-invasively collected fecal samples, hereafter we compare and discuss the possible reasons why gut symbiont richness and prevalence differ between and within primate hosts. To avoid potential effects of seasonality[35–37] and altitudinal gradients[32], fecal samples were collected during a very narrow temporal window from social groups living at similar altitudes; thus, any intraspecific differences should reflect the effects of habitat type. Despite the expected difference between species (with yellow baboons harboring a greater gut symbiont richness compared to the Udzungwa red colobus monkeys), within species, Udzungwa red colobus living in protected forest had a higher level of gut symbiont richness than those living in unprotected forest; this intraspecific difference confirms our previous results for gut bacteria[4,38].

Although they have been evolving with their hosts for millions of years, until recently protozoan and metazoan gut symbionts have been mainly perceived as pathogens, considered harmful rather than integral to gut homeostasis and host health. However, since gut communities have now been recognized as one of the keystones of the human biome and beneficial against inflammatory mechanisms by manipulating the host immune system[39,40], they are being considered relevant to the conservation status of their hosts[20]. This paper contributes to the growing body of knowledge revealing micro biodiversity loss in degraded versus intact habitats[4,9,38], as well as the complex interplay between hosts, symbionts and/or microbiota[21,41–43].

None of the observed taxa of protozoans and metazoans found in this study were new to non-human primates[44], having been noted previously for other red colobus [Ugandan red colobus, *Piliocolobus tephroscelesles*[45,46]; eastern black-and-white colobus *Colobus guereza* and Angolan black-and-white colobus *C. angolensis*[47]] or other baboons [olive baboons, *Papio anubis*[48]; guinea baboons, *Papio hamadryas papio*[49]; yellow baboons, *Papio cynocephalus*[50]]. We found yellow baboons had a higher gut symbiont richness and prevalence than Udzungwa red colobus. Although highly speculative because accurate data are not available, we believe that group density [i.e. number of individuals per group[51,52]] is unlikely to be responsible for this result, as predicted in other animal hosts[53,54]. In fact, yellow baboons and Udzungwa red colobus populations in this study area are known to have comparable group sizes (approximately 40–60 individuals per social group). Body size has also been purported to explain gut symbiont richness and prevalence[55,56], and since yellow baboons are larger than red colobus, this morphological difference could partially explain the higher richness in baboons. However, the two primate

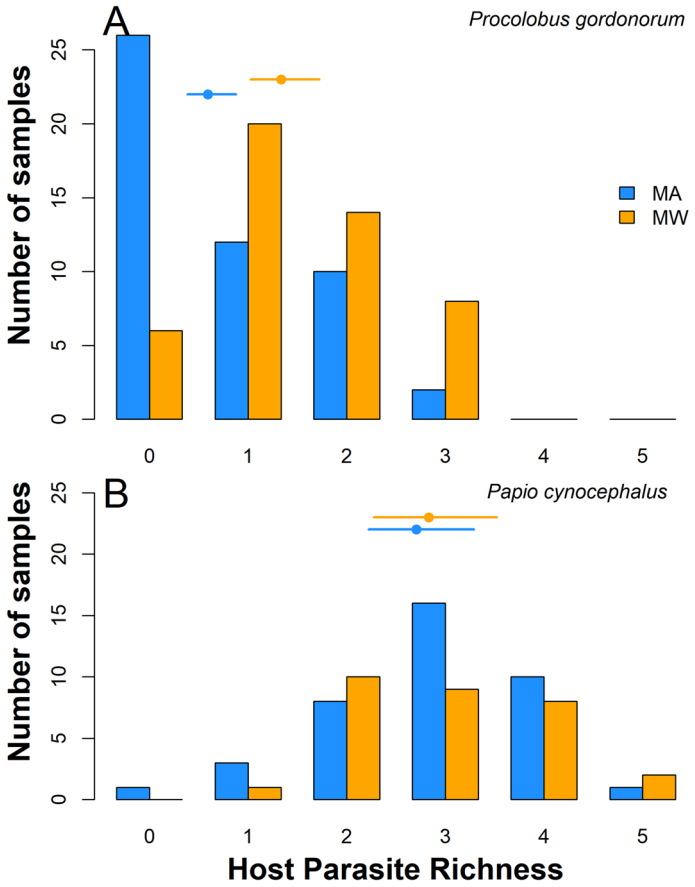


Figure 3. Histogram of the observed gut symbiont richness for two host species, Udzungwa red colobus (*Procolobus gordonorum*) and yellow baboons (*Papio cynocephalus*), in two forests within the Udzungwa Mountains of Tanzania: protected Mwanihana (MW) and unprotected Magombera (MA). Dots represent the expected mean as computed by the Poisson GLM, horizontal bars are the 95% confidence intervals of the mean.

	Estimate	Se	Z value	Pr(>|z|)
Intercept[a]	− 0.274	0.162	− 1.612	0.0907
Species[b]	1.329	0.188	7.081	< **0.0001**
Forest[c]	0.680	0.201	3.391	**0.0007**
Forest × species	− 0.636	0.245	− 2.592	**0.0095**

Table 1. Results of a Poisson Generalized Linear Model (GLM) comparing host symbiont richness (i.e. number of different parasite taxa per fecal sample, including all metazoans and *Balantioides* sp.) between forest type (protected and unprotected), host species (yellow baboon and Udzungwa red colobus) and altitude. Statistically significant values are highlighted in bold. Estimates for qualitative variables are provided as mean difference from the reference (Intercept) while for quantitative variables as average unit increases. Reference levels: [a]Magombera forest (MA) and Udzungwa red colobus (RC); [b]YB: yellow baboon; [c]MW: Mwanihana forest.

species also have very different behavioral traits that could account for the differences in gut symbiont diversities. The most likely driving forces here are diet and time spent on the ground, given the differences in specific gut symbionts' prevalence. The Udzungwa red colobus are predominantly arboreal, occasionally descending to the ground, while yellow baboons spend much of their time travelling on the forest floor and between nearby villages, where they feed on crops and/or on human food waste. These lifestyle differences would explain the higher prevalence of trophically transmitted gut symbionts like spirurid nematodes and dicrocoeliid trematodes in yellow baboons compared to red colobus: both these symbionts are transmitted indirectly through the ingestion of an intermediate host (often arthropods)[44], and baboons consume more terrestrial arthropods in their diet than the mainly leaf-eating Udzungwa red colobus monkeys[51,52]. Similarly, fecal-orally transmitted protozoans from contaminated soil (e.g., *Entamoeba coli*, *Entamoeba* sp., *Iodamoeba buetchlii*), and associated

	Predictor	Estimate	Se	Z-value	Pr(>|z|)
Strongyloides sp.	Intercept[a]	− 1.153	0.331	− 3.481	0.0005
	Species[b] (YB)	3.322	0.623	5.331	**< 0.0001**
	Forest[c] (MW)	1.754	0.448	3.914	**< 0.0001**
	Forest (MW) × species (YB)	− 2.536	0.829	− 3.059	**0.0022**
Trichuris sp.	Intercept[a]	− 0.945	0.315	− 2.999	0.0027
	Species[b]	− 1.225	0.615	− 1.992	**0.0463**
	Forest[c]	0.945	0.427	2.211	**0.0271**
	Forest × species	0.819	0.775	1.058	0.2903
Strongylid nematodes	Intercept[a]	− 2.442	0.521	− 4.411	< 0.0001
	Species[b]	4.147	0.685	6.059	**< 0.0001**
	Forest[c]	0.675	0.663	0.598	0.3090
	Forest × species	− 1.833	0.883	− 1.629	0.0380
Spirurid nematodes	Intercept[a]	− 3.892	1.010	− 3.853	0.0001
	Species[b]	2.537	1.085	2.338	**0.0194**
	Forest[c]	0.042	1.429	0.029	0.9600
	Forest × species	− 1.326	1.654	− 0.802	0.4226
Balantioides sp.	Intercept[a]	− 1.815	0.408	− 4.454	< 0.0001
	Species[b]	3.170	0.569	5.574	**< 0.0001**
	Forest[c]	0.349	0.550	0.634	0.5260
	Forest × species	0.494	0.911	0.542	0.5880

Table 2. Results of Generalized Linear Models (GLMs) of the prevalence of common protozoan and metazoan symbionts (i.e. number of infected individuals of the whole number of samples examined) in relation to host species, Udzungwa red colobus (*Procolobus gordonorum*) and yellow baboons (*Papio cynocephalus*), inhabiting two contrasting forests (degraded Magombera and intact Mwanihana) and altitude. Statistically significant values are highlighted in bold. Estimates for qualitative variables were calculated as the mean difference from the reference (Intercept) while for quantitative variables as mean unit increases. Reference levels: [a]Magombera forest (MA) & Udzungwa red colobus (RC); [b]YB: yellow baboon; [c]MW: Mwanihana forest.

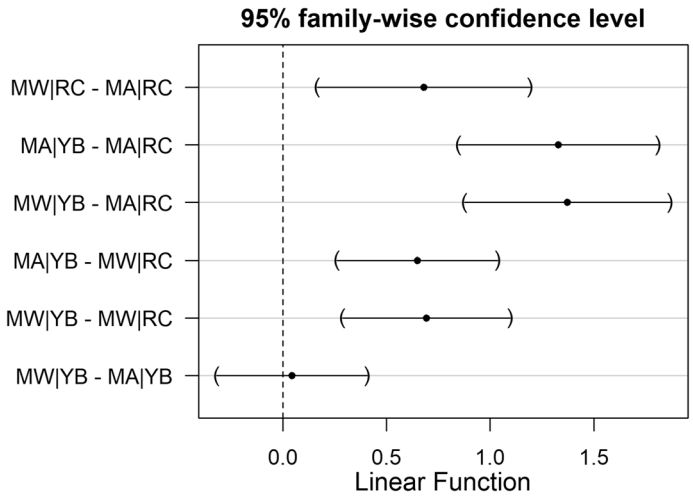


Figure 4. Pairwise comparisons (using Tukey's HSD for the Poisson GLM) of gut symbiont richness for two host species: Udzungwa red colobus (RC on y-axis) and yellow baboons (YB) living in protected Mwanihana (MW) and unprotected Magombera (MA) forests within the Udzungwa Mountains of Tanzania. On the x-axis the estimated mean values and their 95% confidence intervals (CI) are shown for the estimated difference between group's parameters tested in each pairwise comparison. On the y-axis the pairwise comparisons are shown. Black dots represent the average difference and horizontal bands represent the 95% CI.

with domestic animals (*Blastocystis* sp.) were only found in yellow baboons. Another protozoan transmitted through contaminated water (*Balantioides* sp.), as well as two nematode taxa with soil-dwelling free-living stages acquired through the skin (*Strongyloides* sp. and strongylids) were also found significantly more frequently in yellow baboons than red colobus. That these taxa are more likely to be found in terrestrial rather than arboreal hosts has also been observed intraspecifically for chimpanzees *Pan troglodytes*; i.e. individuals that used the ground more frequently for locomotion were more infected[57].

The only difference in gut symbiont prevalence noted here that is not explained by the arboreal-terrestrial lifestyle was for *Trichuris* sp. Although this taxon is also soil-transmitted, this genus was more prevalent in arboreal Udzungwa red colobus rather than baboons. Another larger study specifically focused on on *Trichuris* sp. in non-human primates was also unable to identify the factors responsible for prevalence in particular primate species, but terrestriality was not one of them[58]. However, *Trichuris* sp. do appear to prefer dense shade which may explain the discrepancy[59], since red colobus tend to live in deeper shader forest, while baboons spend much of their time on forest edges. The same peculiarity of *Trichuris* sp. may also explain why in both species, this genus was more prevalent in MW than MA.

Studies on the effect of human disturbance on intraspecific variation of gut symbiont richness and prevalence in wild animals still present very mixed results[60]. For example, several studies conducted on primates have found lower gut symbiont richness in intact, undisturbed habitats compared to disturbed and degraded ones (Tana River red colobus, *Procolobus rufomitratus* and mangabey, *Cercocebus galeritus galeritus*[54]; lion-tailed macaque, *Macaca silenus*[61]; redtail guenon, *Cercopithecus ascanius*[62]). However, the same studies were unable to reveal correlations between richness and habitat type on other primate populations (red colobus, *Piliocolobus tephrosceles* and black-and-white colobus, *Colobus guereza*[62]). Similarly, no correlation was found between prevalence and habitat type (lion-tailed macaque, *Macaca silenus*[61]; Tana River red colobus, *Procolobus rufomitratu*[54]; red colobus, *Piliocolobus tephrosceles* and black-and-white colobus, *Colobus guereza*[62]). On the other hand, similar to our results, in the black howler monkey (*Alouatta pigra*) gut symbiont reduction was clearly associated with habitat degradation[63], as well as in other mammals such as Australian skinks *Lampropholis guichenoti*[64] and several sigmodontinae rodent species (*Akodon cursor*, *A. montensis* and *Oligoryzomys*) from Brazil[65].

The discrepancy in finding correlations between gut symbiont richness and habitat type within each primate species investigated might be explained by the different ecological adaptations of each species. It is likely that host species dependent on forest integrity for its diet like the leaf-eating red colobus are influenced differently from human disturbance. In fact, human presence and intensified activities in the unprotected MA forest may have played a critical role in shaping habitat features over time and consequently reducing gut symbiont presence and potential transmission. For example, logging may have changed the forest structure by increasing forest edges and fragmentation of canopy cover may have promoted increased exposure to wind and sun radiation facilitating drier conditions and reducing survival of free-living stages of gut symbionts, resulting in lower infections of individuals living in such altered habitats. Although speculative, the lower contamination of gut symbionts in degraded habitats, such as the small lowland forest fragment of Magombera, could also potentially be due to the proximity of sugar cane plantations[66] and cultivated fields which are often treated with pesticides, fertilizers and anti-helminthics.

Although not true for all animal species, the endangered Udzungwa red colobus monkeys have now been shown to suffer biodiversity loss at multiple levels in degraded, unprotected habitats. Not only are there lower population densities of this species in MA[30,67], but lower taxonomic and functional diversity of gut bacteria were found in individuals living in this unprotected forest[4,38]. Furthermore, the present study confirms that gut symbiont richness is reduced in red colobus inhabiting MA. If such associations are confirmed in other specialist feeders, it is plausible that gut communities could be used as a tool for evaluating individual health and the conservation status of a species, especially in the case of threatened taxa where non-invasive fecal sampling is necessary[68]. However, further studies are required to identify potential interactions between gut components and between these components and host health before these relationships can be used as biomarkers for the wellbeing of individuals and species.

Methods

Sampling site and animals. The Udzungwas represent the southernmost range of the Eastern Arc Mountains, occupying an area of approximately 19,000 km^2 (7° 40′ S–8° 40′ S and 35° 10′ E–36° 50′ E; Fig. 5), forming a mosaic of moist forest blocks, interspersed with a matrix of naturally drier habitats, but also croplands and settlements. Rainfall averages 1,500–2,000 mm per year concentrated in two periods: November–December and March–May. Altitude ranges from 270 m a.s.l. (Kilombero valley in the eastern side) to 2,576 m a.s.l. (Mount Luhomero). The protected Mwanihana (MW) and unprotected Magombera (MA) forests were chosen as study sites (Fig. 5; Table 3; see[69] for details). In the past 50–60 years, these two forests have been gradually separated from each other by at least 6 km of fields and villages[70]. Troops of *Procolobus gordonorum*, the endemic Udzungwa red colobus monkeys, and *Papio cynocephalus*, the yellow baboon live sympatrically in both forests. The Udzungwa red colobus are arboreal and mainly leaf-eating, whereas yellow baboons are terrestrial and omnivorous, feeding regularly on sugar cane as well as human organic waste[51].

Sample collection. Fecal pellets were collected from 12 red colobus social groups (N = 98 individuals), six (N = 48) from MW, and six (N = 50) from MA. For yellow baboons, samples were collected from five social groups (N = 69), three (N = 30) from MW and two (N = 39) from MA. Most baboon samples were found close to forest edges where baboons regularly crop-raid (especially those from MA). Because red colobus and baboons live in large social groups (~ 40 individuals) and are highly elusive, fecal samples could not be assigned to individuals. However, to avoid any potential re-sampling of the same individual, samples were collected non-inva-

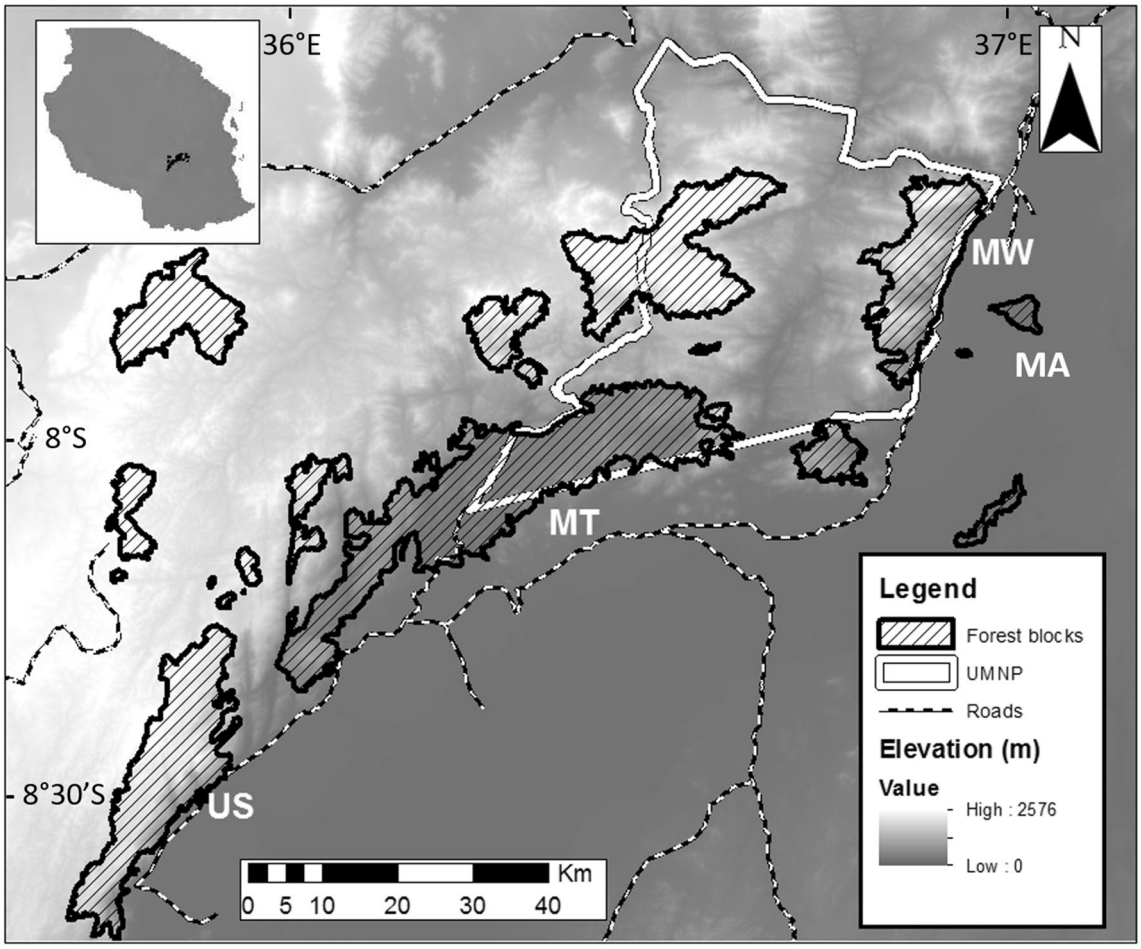


Figure 5. Map of the Udzungwa Mountains in Tanzania showing various forest blocks, including the protected Mwanihana (MW) and unprotected Magombera (MA) considered in this study. The borders of the Udzungwa Mountain National Park (UMNP) are highlighted in white (Modified from Araldi et al. 2014).

	MW	MA
Protection level and human presence	National Park (since 1992); several villages along the eastern edge	No formal protection, with a long and complex history of failed attempts of protection; the forest is reducing in size and frequently entered for collecting firewood and hunting
Size (km^2)	150.6	11.9
Altitude (m)	351–2,263	269–302
Forest type	Lowland, semi-deciduous, sub-montane and montane evergreen forest, including upper montane, bamboo-dominated forest	Ground-water lowland evergreen forest, remaining patches of once continuous forest; surrounded villages and intensive agriculture

Table 3. Characteristics of the protected Mwanihana (MW) and unprotected Magombera (MA) forests in the Udzungwa Mountains of Tanzania. Forest size and altitude from Marshall et al. (2010).

sively during a single defecation event from each group. Social groups sampled during the same day were located at least 2 km apart (more details for sampling procedures in[4,70,71]).

All samples were collected in 2016 during the same 4-week temporal window to avoid potential seasonal effects[36,37]. An aliquot of 2 g of fresh feces was preserved in 10 ml of 10% neutral buffered formalin and stored at ambient temperature (20–25 °C) until transport to the Fondazione E. Mach in Italy where they were kept at 4 °C before definitive shipment of aliquots to the University of Veterinary and Pharmaceutical Sciences in Brno, Czech Republic for parasitological analysis.

Protozoan and metazoan symbionts examination. Host blood, tapeworm proglottids and adult nematodes that were clearly visible by eye in fecal pellets were noted in the field prior preserving the 167 samples. As recommended for parasitological analyses of non-human primates[44,72], we used both modified Sheather's flotation and fecal sedimentation techniques to identify symbionts. First we extracted and weighed sediment

from the whole sample, then examined 2 ml of fecal suspension with optical microscopy at 400× magnification (Olympus CX40) for gut symbiont identification. Based on morphological characteristics[44] eggs and cysts were quantified for each symbiont taxon and the number of eggs or cysts per gram of sediment were calculated according to the following formula: n = N/m, where n = number of eggs/weight of sediment (g), N = number of eggs in examined amount of sediment and m = weight of examined sediment (g). Lastly, using what of the fecal pellet after extraction of sediment, adult and larval nematodes were collected using the 'gauze-washing' method[73] and observed via stereo microscopy at 8–40× magnification (OLYMPUS SZ51).

Data analyses. Three standard indices of gut symbiont infection were estimated and used as dependent variables in the subsequent regression models:

1. gut symbiont prevalence (i.e. number of infected samples out of the total number of samples examined);
2. gut symbiont richness (i.e. number of different symbiont taxa per sample);
3. gut symbiont egg or cyst shedding intensity (i.e. number of eggs or cysts per gram of fecal sediment for *Strongyloides* sp. and *Balantioides* sp. only).

To assess if the prevalence of each symbiont varied with host species, forest type or the interaction of these two factors, a Generalized Linear Model (GLM) with Binomial distribution and logit link was carried out. Similarly, the association of symbiont richness with primate species, forest type or their interaction was studied using a GLM with Poisson distribution and log link. Because some protozoans (e.g. amoebas and *Blastocystis*) are difficult to detect with optical microscopy[74,75], the models including symbiont richness were run twice: once with all protozoan taxa, and a second time excluding rare taxa, i.e. where rare is defined as being present in fewer than 10 samples (*Iodamoeba buetschlii*, *Entamoeba coli*, *Entamoeba* spp., *Blastocystis* sp.). Since results from a previous study on the same population of red colobus monkeys revealed that altitude may affect gut symbiont richness in this species[32], the sampling design was limited to social groups of both species at low altitude (less than 450 m a.s.l.). The altitudinal range in the present study was much narrower (216–441 m) than in the previous investigation (216–1535 m[32]). Therefore, we consider inclusion of altitude in the models by computing the Akaike Information Criteria (AIC) values for each model with or without altitude and decided not to include it (results in Supplementary Information). Gut symbiont diversity measures such as prevalence and richness may depend on infection shedding[76]. Thus, in infected fecal samples, a linear model (LM) was used to assess if the log-transformed egg or cyst shedding intensity for each individual host was associated with primate species or forest type (and their interaction), or altitude. Altitude was standardized (subtracted by its mean and divided by its standard deviation, so to have mean 0 and standard deviation 1). Assessment of the appropriateness of statistical assumptions for each model was carried out by computing overdispersion for GLM and graphically inspecting model residuals[77]. All analyses were carried out in R environment (version 3.5.1; R Core Team 2018).

Ethics statement. The authors confirm they did not interact with or disrupt any of the primate species surveyed in any way. Fecal sample collection was non-invasive, without direct contact or interaction with the animals. Highly trained fieldworkers strictly adhered to the 'Code of Best Practices for Field Primatology' published by the International Primatological Society (IPS) as well as the 'Principles for the Ethical Treatment of Primates' of the American Society of Primatologists (ASP). Data collection complied with legal requirements and laws governing wildlife research in Tanzania. Research permits (2016-267-ER-2009-49) were obtained through the Tanzania Commission for Science and Technology (COSTECH), Tanzania Wildlife Research Institute (TAWIRI) and Tanzania National Parks (TANAPA).

Data availability

Data are available in the Figshare repository at https://figshare.com/s/3fe0e6a0bada2a508ad3.

Received: 20 March 2020; Accepted: 20 May 2020
Published online: 02 July 2020

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Acknowledgements

We thank the Tanzania Commission for Science and Technology (COSTECH), Tanzania Wildlife Research Institute (TAWIRI) and Tanzania National Parks (TANAPA) for granting us permissions to conduct the study (COSTECH Permit n. 2016-267-ER-2009-49). We also thank the rangers of Udzungwa Mountains National Park, A. Mwakisoma and R. Laizzer for providing invaluable field assistance and R.M. Cameira, A. Stryková and A. Gördes for parasite identification. Data collection and analyses were funded by the European Union's Horizon 2020 research and innovation programme under the Marie Sklodowska-Curie Grant agreement No 752399 (Project WILDGUT).

Author contributions

C.B. and H.C.H. conceived and designed the study and obtained the funding. C.B. and F.R. collected field data and samples. C.B., B.P. and D.M. performed and supervised the parasitological analyses. M.M. and R.R. conducted statistical analyses. C.B. wrote the first draft of the manuscript. All other authors actively reviewed and contributed to the manuscript.

Competing interests

The authors declare no competing interests.

Additional information

Supplementary information is available for this paper at https://doi.org/10.1038/s41598-020-67959-7.

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Mason, B., Piel, A.K., Modrý, D., Petrželková, K.J., Stewart, F.A., Pafčo, B.

2022b

Association of human disturbance and gastrointestinal parasite infection of yellow baboons in western Tanzania.

PLoS ONE 17, e0262481. https://doi.org/10.1371/journal.pone.0262481

PLOS ONE

RESEARCH ARTICLE

Association of human disturbance and gastrointestinal parasite infection of yellow baboons in western Tanzania

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Citation: Mason B, Piel AK, Modrý D, Petrželková KJ, Stewart FA, Pafčo B (2022) Association of human disturbance and gastrointestinal parasite infection of yellow baboons in western Tanzania. PLoS ONE 17(1): e0262481. https://doi.org/10.1371/journal.pone.0262481

Editor: Nicoletta Righini, Universidad de Guadalajara, MEXICO

Received: June 7, 2021

Accepted: December 24, 2021

Published: January 12, 2022

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Data Availability Statement: All relevant data are within the manuscript and its Supporting information files. All raw data is publicly available through two datasets accompanying this manuscript as supplementary tables. The first, 'S2 Table' details the results of our coproscopic analysis of faecal samples, while the second, 'S3 Table', details the results from our transects to assess human disturbance.

Funding: The authors received no specific funding for this work.

Abstract

Human disturbance is an ongoing threat to many wildlife species, manifesting as habitat destruction, resource overuse, or increased disease exposure, among others. With increasing human: non-human primate (NHP) encounters, NHPs are increasingly susceptible to human-introduced diseases, including those with parasitic origins. As such, epidemiology of parasitic disease is becoming an important consideration for NHP conservation strategies. To investigate the relationship between parasite infections and human disturbance we studied yellow baboons (*Papio cynocephalus*) living outside of national park boundaries in western Tanzania, collecting 135 fresh faecal samples from nine troops occupying areas with varying levels of human disturbance. We fixed all samples in 10% formalin and later evaluated parasite prevalence and abundance (of isotrichid ciliates and Strongylida). We identified seven protozoan and four helminth taxa. Taxa showed varied relationships with human disturbance, baboon troop size and host age. In four taxa, we found a positive association between prevalence and troop size. We also report a trend towards higher parasite prevalence of two taxa in less disturbed areas. To the contrary, high levels of human disturbance predicted increased abundance of isotrichid ciliates, although no relationship was found between disturbance and Strongylida abundance. Our results provide mixed evidence that human disturbance is associated with NHP parasite infections, highlighting the need to consider monitoring parasite infections when developing NHP conservation strategies.

Introduction

Parasites can harm host species through a variety of ways, from altering social behaviours [1] to diminishing host physical health and consequently reducing reproductive and foraging

Competing interests: The authors have declared that no competing interests exist.

success [2]. Parasite infection can further cause damage to neural tissue, reduce fitness, and can even prompt population decline [1, 3], with some infections being fatal to their host [4, 5]. Despite this, parasitic infections in wild animals are often asymptomatic [6, 7], with many hosts coevolving with their parasites overtime [8] and some now believed to be commensals or even mutualists that benefit host physiological functioning, such as aiding digestion [9, 10]. The capacity of parasites to be detrimental to host health may be exacerbated in wild animals that live in fragmented populations or in close contact to human settlements [11], through either increased occurrence of clinical infections [12] or loss of symbiont diversity [10]. This highlights the need to consider the association between human disturbance and parasite infections in wild animals.

Human disturbance has the potential to influence parasite infection of wildlife hosts through multiple indirect mechanisms, such as inducing chronic stress through increased human (predator) encounter rates, urbanisation and noise pollution [13–15], with cascading effects resulting from immunosuppression [16]. Reduced food availability, resulting from habitat disturbance, increases susceptibility to parasites by compromising host nutrition [17, 18]. Additionally, human disturbance may influence parasite infections of wild hosts through transmission from domestic animals, with ongoing agricultural expansion, driven by growing human food demands [19], increasing wildlife to livestock exposure [20]. Livestock proximity has been found to correlate with increased parasite infection in a range of species [21]. Humans can also transfer pathogens to wild animals through reliance on common resources, such as water sources, with poor human sanitation heightening this potential [22, 23]. Transmission from humans is even more likely for non-human primates (NHPs) [6, 24], with their close phylogenetic relationship resulting in numerous shared parasite taxa [21]. Expanding human settlements and fragmentation of wildlife habitat increases human:NHP interactions [6], and thus transmission risk, with forest fragmentation previously linked to increased gastrointestinal parasite infections in western chimpanzees (*Pan troglodytes verus*) [25], among others.

While there are many proposed mechanisms of how human disturbance can influence wildlife hosts, mechanisms are typically taxa specific. *Papio* are known to adapt to anthropogenic environments relatively well [26], in part due to being dietary generalists [27, 28], meaning habitat disturbance is unlikely to influence parasite infections through compromised nutritional status in these hosts. However, higher parasite richness in olive baboons (*P. anubis*) has previously been linked with their foraging in anthropogenic habitats [29]. Despite being one of the most terrestrial primates, *Papio* still exhibit arboreal behaviours [30], meaning timber extraction may influence host behaviour. Anthropogenically induced behavioural shifts can increase host exposure risk to parasite infections and consequently shift transmission dynamics, with intensity of timber extraction one factor of human disturbance previously associated with nematode prevalence and infection risk (assessed through a specific index) amongst red colobus (*Piliocolobus tephrosceles*) [31]. Additionally, through either chronic stress or shared parasite taxa, human presence may also influence parasitic infections in *Papio*, as found in lion-tailed macaques (*Macaca silenus*), where increased parasite prevalence and species richness were explained by human presence [17].

Aside from human disturbance, other factors can also influence parasite transmission dynamics. Group size is positively associated with parasite infection in some populations [32]. A variety of mechanisms are proposed for this, including increased exposure resulting from higher animal densities [32] as well as resource competition increasing susceptibility to infection [33, 34]. Age is also associated with parasite infections, with exposure risk and susceptibility to infection influenced by both behavioural and physiological differences between juvenile and adult hosts [35].

For the current study, we focused on nine troops of baboons living within the Basanza Forests of the Uvinza district and Tongwe East and West Forest Reserves, in western Tanzania. The gastrointestinal parasites of baboons are widely documented across *Papio* geographic distribution [29, 36–39]. Gastrointestinal parasites of baboons in Gombe and Mahale Mountains National Parks (Tanzania) have previously been described, identifying nine metazoan and one protozoan species [40]. Despite this extensive documentation of *Papio* parasite infections, little research has focused on the potential impact of human disturbance on these infections. Previously, gastrointestinal symbiont richness in yellow baboons (*Papio cynocephalus*) showed no variation between troops inhabiting protected and unprotected forests in central Tanzania [10]. We compare gastrointestinal parasites of yellow baboons occupying habitat along a gradient of human disturbance in western Tanzania. We investigated troops of differing group size, comprised of both juvenile and adult individuals. We hypothesised that habitat disturbance would be associated with higher prevalence of parasite taxa, resulting from increased transmission risk of previously documented human: NHP shared parasites. We also predicted human disturbance to be associated with higher parasite abundance, due to behavioural shifts and human presence increasing parasite exposure and susceptibility, respectively.

Material and methods

Study site

We conducted sampling in Tongwe East and West Forest Reserves within the Greater Mahale Ecosystem and Basanza Forests (Uvinza District) within the Masito-Ugalla Ecosystem, western Tanzania. We selected the Issa Valley (-5.5, 30.54) as a low disturbance area and the outskirts of Uvinza (-5.1, 30.39), a town of >10,000 people ca. 50 km from Issa Valley, as a high disturbance area (Fig 1). In total, we surveyed an area 146 km^2: ca. 45 km^2 near Issa Valley and ca. 101 km^2 near Uvinza. Issa valley is characterised by large expanses of miombo woodland, separated by broad valleys with steep slopes, lined with riparian forests and intermittent grasslands [41, 42]. Uvinza hosts a sprawling town, surrounded by a similar habitat mosaic interspersed

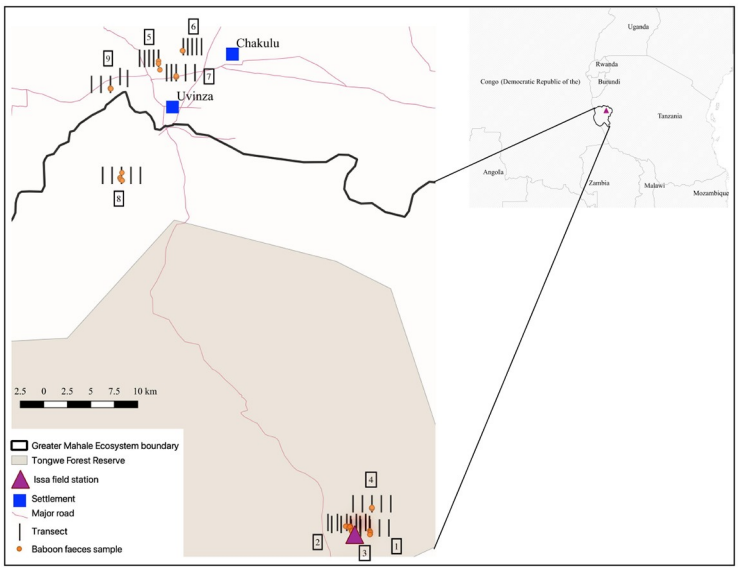


Fig 1. Study area in western Tanzania. Visual mapping of Tongwe Forest Reserve [45] in Western Tanzania, overlayed with GPS log of faecal collections and transect locations for nine yellow baboon troops, generated in QGIS (v 3.8). Group number is indicated by the boxed number adjacent to transect lines.

https://doi.org/10.1371/journal.pone.0262481.g001

Table 1. Sample collection from yellow baboons in western Tanzania.

Sampling Area	Group	Group Size	Samples Collected		
			Total	Adult	Juvenile
Issa Valley	1	13	12	10	2
	2	25	19	19	0
(n = 91)	3	37	19	17	2
	4	21	21	18	3
	8	28	20	19	1
Uvinza District	5	5	4	4	0
	6	7	7	6	1
(n = 44)	7	12	10	8	2
	9	29	23	19	4
TOTAL		**177**	**135**	**120**	**15**

Number of samples collected from yellow baboons in western Tanzania split by all relevant factors (sampling area, group, and estimated age).

https://doi.org/10.1371/journal.pone.0262481.t001

with small-scale agriculture and logging sites [43]. Average altitude of sample collection was 1515.5 m in Issa Valley and 1051.3 m in Uvinza, with yearly rainfall around 1000 mm in both areas [41, 42]. Temperature ranges from 11 to 35 ˚C in Issa Valley [41] and from 20 to 30 ˚C in Uvinza [44].

Faecal sample collection

We collected all faecal samples between April and May 2018, spanning the transitional period between the wet (November-April) and dry (May-October) seasons, with dry months defined as those with <60 mm of rainfall [42]. We collected 135 faecal samples of baboons from nine troops (Table 1). One troop (group 1) was habituated and we used individual identifications to ensure no individual was repeatedly sampled, for other troops we collected all faecal samples within a 120 min time period, assuming the limited timespan prevented repeated sampling [46]. We estimated group size by counting all visible individuals prior to sampling, assuming the entire group was counted. We collected samples as soon after defecation as possible (when baboons vacated the immediate vicinity) and stored in sealed sampling pots, with only the central faecal matter being collected to avoid environmental contamination [37, 39]. We recorded a GPS location for each sample and assigned baboon age categories based on size of faeces (juvenile <2 cm faecal diameter; adult >2 cm faecal diameter) [47]. For smaller troops (<15 individuals) we sampled at least 80% of individuals and at least 50% in larger troops (>20 individuals), based on a recommended sample size and gastrointestinal parasite prevalence in olive baboons [18, 39]. Within 180 min of initial collection and storage, we mixed each sample and fixed ca. 3 g of faeces with 10% formalin, storing at room temperature before shipment to the University of Veterinary Sciences, Czech Republic, for analyses.

Assessing human disturbance

Although we selected two localities with presumably low (Issa Valley) and high (Uvinza District) human disturbance, we still evaluated the human impact directly in the areas occupied by the baboon groups at the time of collection. We assessed human disturbance through a general disturbance index adapted from Barelli et al. [48]. We established five northward orientated 2-km long transects for each sampled troop. Transects were spaced 1 km apart, covering a total area of 8 km^2 per troop (Fig 1). We recorded all evidence of human disturbance within a five-metre strip of transects, including, but not limited to, roads, livestock, honey production



Fig 2. Human disturbances observed in the Basanza Forests miombo woodland surrounding Uvinza, Tanzania. (a) Crop agriculture; (b) Livestock agriculture; (c) Cutting signs; (d) Constructional timber production; (e) Charcoal production; (f) Vehicle track.

https://doi.org/10.1371/journal.pone.0262481.g002

and litter (Fig 2). All signs of tree cutting were also recorded, including cut shrubbery, trees, and any machete marks. We generated transect locations systematically from the faecal collection GPS centroid of each troop, which formed the middle point of the central transect (Fig 1). If natural barriers (e.g., rivers) prevented transect access, we walked additional transects between those that were accessible, ensuring we conducted five transects per troop. If a transect could not be finished due to impassable terrain, the distance completed was used to provide a proportional score. We calculated a mean transect score for each troop then pooled groups by disturbance level based on naturally forming categories: low (< 10 signs) or high (> 300 signs) (Fig 3). Overall, the disturbance levels correspond to the locality which baboon troops inhabit. Areas with high human disturbance are within the Uvinza district (troops assigned high-disturbance level), while low disturbance was observed within the Issa Valley area (groups with low-disturbance level). Interestingly, one group (Group 8, see Fig 3) ranged

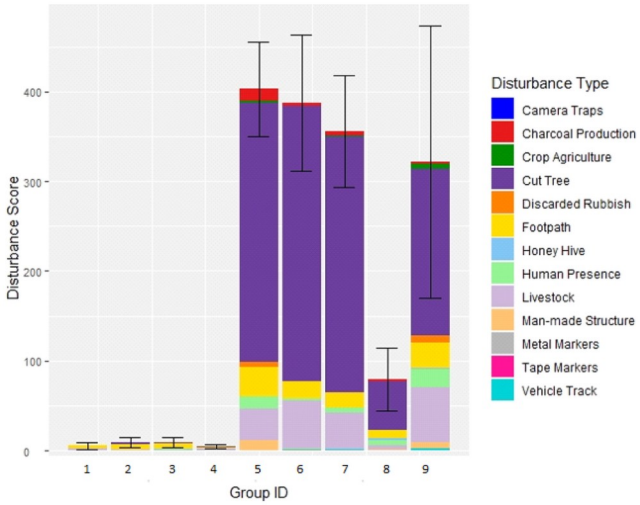


Fig 3. Human disturbances observed in the habitat of yellow baboon troops in western Tanzania. Average human disturbance score, calculated through five transects, recorded in the habitat of nine troops of yellow baboons in western Tanzania, with fill colour depicting the type of human disturbances encountered.

https://doi.org/10.1371/journal.pone.0262481.g003

between these two localities and did not fall into these disturbance categories, (79 signs); we assigned this area with medium disturbance levels, testifying to the declining human activities toward the Issa Valley.

Faecal sample analysis

We used coproscopic diagnostics by light microscope (Olympus CX40) to determine gastrointestinal parasite diversity and abundance (a proxy for infection intensity) [49]. We diluted samples with water and filtered through gauze before halving sediment to re-fix half (up to 10 ml) in 10% formalin and suspend half (up to 50 ml) in sodium chloride solution [50]. We used the suspended solution to quantify strongylid nematode eggs per gram (EPG), using Mini-FLOTAC apparatus (x100 magnification) and recounting formula. We used the re-fixed sediment to evaluate parasite diversity by Sheather's flotation [51], documenting all parasite taxa, using cyst, trophozoite and egg morphology for identification [6, 52]. We also examined 0.2 ml of the sediment directly for protozoa diversity (x400 magnification) and evaluation of isotrichid ciliate cyst and trophozoite abundance (CPG = ciliates per gram) [53]. We quantified strongylids and isotrichid ciliates because of their possible pathogenic potential [54].

Statistical analysis

We performed all statistical analyses using the R statistical interface, R v.3.2.2 [55]. We investigated factors influencing parasite infection, with parasite presence/absence and abundance (measured as EPG/CPG) as response variables, using Generalized Linear Mixed Models (GLMMs). We ran GLMMs using Ime4 package [56] with binomial distribution for presence/absence data and negative binomial distribution for abundance data, to account for the aggregated distribution of parasites within-host population. First of all, our aim was to evaluate the impact of human disturbance level (three categories: low in Issa valley, high in Uvinza district and medium in between these two localities) and further impact of group size and age on the presence/absence and/or abundance of yellow baboon parasites, therefore we set these as explanatory variables. Lastly, as the medium disturbance level was represented by only one

group, we set the group affinity as a random factor. We ran a separate model for each parasite and log-transformed the abundance data. Finally, we used Tukey post hoc tests to evaluate differences among levels of factorial explanatory variables in case of disturbance level. We did not use parasite richness to test impact of human disturbance, group size or age, as some parasite taxa (e.g., strongylids and spirurids) comprise multiple species, which are indistinguishable as egg/cysts, which limits the credibility of parasite richness.

Ethics statement

All protocols were approved by The Animal Welfare and Ethics Steering group at Liverpool John Moores University and adhered to the UK Home Office Animals (Scientific Procedures) Act (ASPA). Research was approved by the Tanzania Commission for Science and Technology (COSTECH), permit no. 2017-320-NA-2011-94, and we collected all samples non-invasively, adhering to Tanzania Wildlife Research Institute (TAWIRI) regulations.

Results

Parasite taxa

From the 135 samples examined, we found a total of six protozoan taxa and four helminth taxa (Fig 4), with prevalence data shown in Table 2. We identified three protozoa to species level: *Entamoeba coli*, *Iodamoeba buetschlii* and *Chilomastix mesnili*; and two protozoan taxa to genus level: *Blastocystis* and *Entamoeba* spp. We also noted unidentified isotrichid ciliates. *Entamoeba coli* was identified to species based on the presence of at least eight nuclei, whilst identified *Entamoeba* with alternative nuclei numbers were grouped as *Entamoeba* spp. [35, 57]. Isotrichid ciliates (95.56% positive) and *Entamoeba* spp. (91.11% positive) were the most prevalent protozoa. Four samples were negative for all protozoa. We identified the four helminth taxa based on egg presence, two to genus: *Strongyloides* and *Streptopharagus*, and two to order/suborder (because of microscopically indistinguishable eggs): Strongylida (later strongylids) and Spirurida (later spirurids). *Streptopharagus*' smaller size compared to other spirurids [58] was used for its distinction. Amongst the helminths, strongylids were the most prevalent, confirmed in all collected samples.

The mean abundance of isotrichid ciliates was 231.5 EPG, SD: 410.6, and of strongylid nematodes 422.5 EPG, SD: 487.2, in all samples.

Association with human disturbance

Our transects confirmed higher occurrences of human disturbance in baboon habitat surrounding Uvinza than in Issa Valley, with medium levels of disturbance encountered between the two sites (Figs 1 and 3, Group 8). While a variety of different types of human disturbance were observed, the majority were signs of tree cutting, with livestock and footpaths also frequently occurring.

All parasite taxa were found across all three disturbance levels. Prevalence of two protist and one helminth taxa were associated with disturbance level. The prevalence of *E. coli* was significantly associated with disturbance level (GLMM: $\chi = 126553.0$, $p < 0.001$), whereby the prevalence was higher in low disturbance areas compared to high ($p < 0.001$). Similarly, the prevalence of *I. buetschlii* was significantly different according to disturbance level (GLMM: $\chi = 13.384$, $p = 0.001$), due to being higher in low disturbance areas compared to both in medium ($p = 0.001$) and (although not significant) in high ($p = 0.066$) disturbance areas. Significant differences were observed in distribution levels according to prevalence of *Streptopharagus* sp. (GLMM: $\chi = 10.106$, $p = 0.006$). Significantly higher prevalence of *Streptopharagus* sp. was

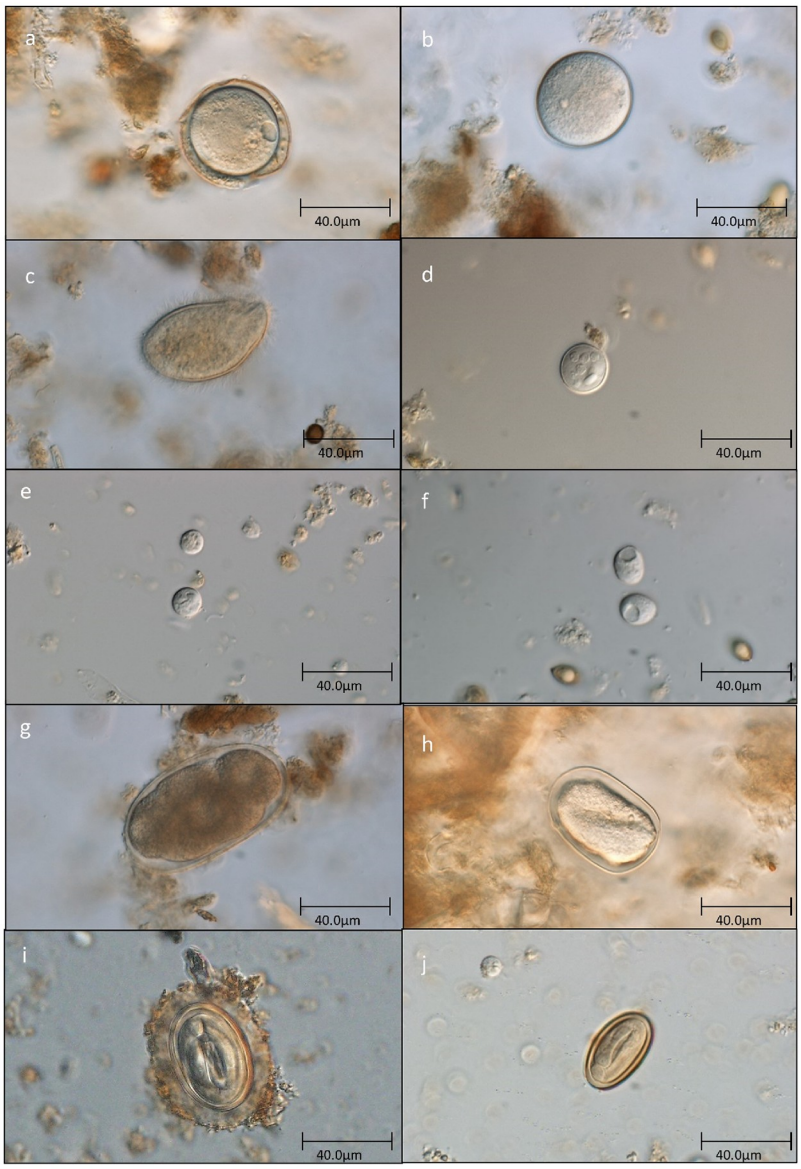


Fig 4. Photos of parasites found in yellow baboons' faeces in western Tanzania. Unidentified isotrichid ciliate cysts: (a) with thick outer protective sheath; (b) without additional protective sheath. (c) Unidentified isotrichid ciliate trophozoites. (d) *Entamoeba coli*. (e) *Entamoeba* sp. (lower cyst) and *Blastocystis* sp. (upper cyst). (f) *Iodamoeba buetschlii*. (g) strongylid egg. (h) *Strongyloides* sp. (i) Spirurid egg. (j) *Streptopharagus* sp.

https://doi.org/10.1371/journal.pone.0262481.g004

observed in medium disturbance areas compared to low disturbance areas ($p = 0.011$). Prevalence of other parasite taxa was not significantly associated with disturbance level (S1 Table). Increasing disturbance did show a positive association with isotrichid ciliate abundance (GLMM: $\chi = 15.8$, $p < 0.001$; for results of post hoc tests see Fig 5). Though no significant association with strongylid abundance was found (GLMM: $\chi = 1.433$, $p = 0.488$; Fig 5).

Impact of group size and age

The prevalence of *I. buetschlii*, *E. coli*, *Blastocystis* sp., and *Streptopharagus* sp. was positively associated with group size (GLMM: $\chi = 14.6981$, $p < 0.001$; GLMM: $\chi = 1024.1$, $p < 0.001$;

Table 2. Prevalence of gastrointestinal parasites of yellow baboons.

	Prevalence (%)			
	Total	Low disturbance	Medium disturbance	High disturbance
	(135)	(71)	(20)	(44)
Isotrichid ciliates	95.56	100.00	100.00	86.36
Entamoeba coli	67.41	76.06	55.00	59.09
Entamoeba spp.	91.11	97.18	95.00	79.55
Iodamoeba buetschlii	70.37	84.51	55.00	54.55
Blastocystis sp.	69.63	63.38	95.00	68.18
Chilomastix mesnili	6.67	7.04	0.00	9.09
Strongylids	100.00	100.00	100.00	100.00
Strongyloides sp.	23.70	32.39	20.00	11.36
Spirurids	22.96	22.54	25.00	22.73
Streptopharagus sp.	44.44	33.80	75.00	47.73

Overall prevalence of gastrointestinal parasites of yellow baboons in Issa Valley and Basanza Forests, western Tanzania, as well as prevalence at each of three disturbance levels. Values in parenthesis indicate number of faecal samples examined.

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GLMM: $\chi = 6.478$, $p = 0.011$; GLMM: $\chi = 5.084$, $p = 0.024$, respectively) (Fig 6). Additionally, juveniles had increased prevalence of *E. coli* (66.6% in adults; 73.3% in juveniles; GLMM: $\chi = 1777.9$, $p < 0.001$) and *Strongyloides* sp. (20% in adults; 53.3% in juveniles; GLMM: $\chi = 7.365$, $p = 0.007$). Prevalence of other parasite taxa was not significantly associated with group size or age (S1 Table).

Group size was significantly associated with isotrichid ciliate abundance (GLMM: $\chi = 6.99$, $p = 0.008$), while strongylids abundance was not (GLMM: $\chi = 0.321$, $p = 0.571$). Age showed no association with abundance of either isotrichid ciliates (GLMM: $\chi = 0.214$, $p = 0.644$) or strongylids (GLMM: $\chi = 0.064$, $p = 0.801$).

Discussion

Western Tanzania is a mosaic of varied land status, from national parks to open areas, with multiple NHP species, including yellow baboons, distributed throughout, including adjacent to human settlements. To assess the relationship between human disturbance and

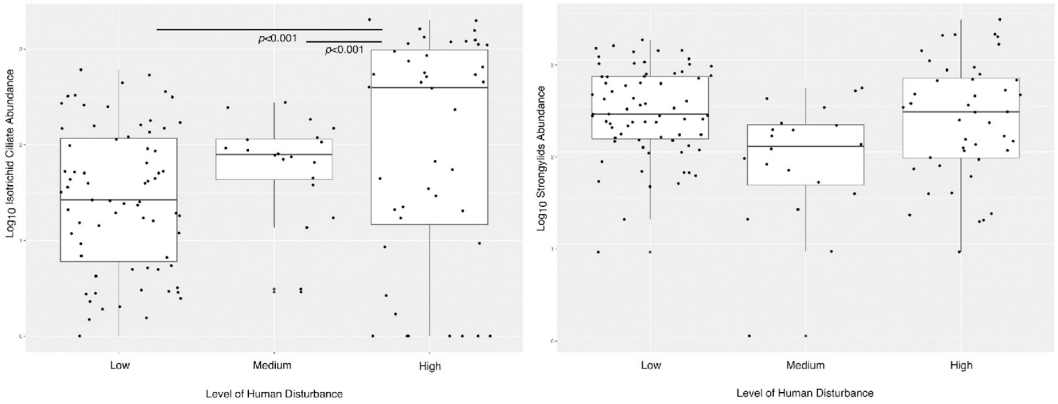


Fig 5. Parasite abundance in yellow baboons in western Tanzania. Plots are split by human disturbance of habitat area and show two parasite taxa: (a) unidentified isotrichid ciliate; (b) strongylids.

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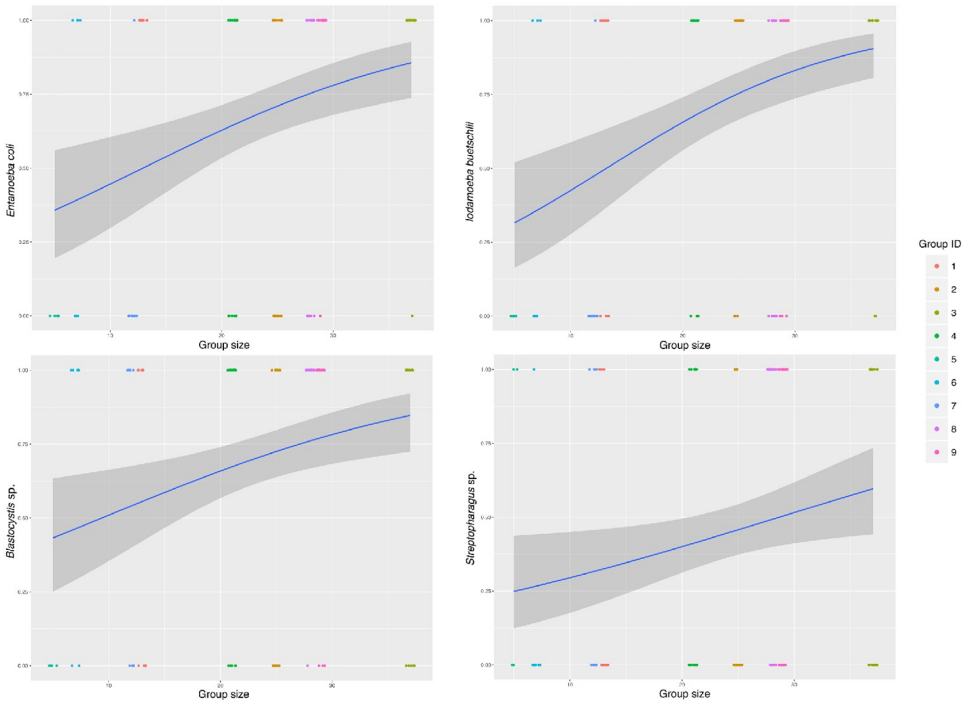


Fig 6. Association between yellow baboon group size and parasite prevalence. Association between group size and parasite prevalence of four parasite taxa amongst yellow baboons situated in western Tanzania.

https://doi.org/10.1371/journal.pone.0262481.g006

gastrointestinal parasite infection of yellow baboons, we examined faecal samples from baboon troops inhabiting areas with different levels of human disturbance. Some troops ranged in intact forest areas with minimal human activities, while others ranged closer to human settlements, with larger disturbance signatures, including tree felling, cattle grazing and charcoal production; in some cases, individuals were observed crop raiding.

In general, all ten parasite taxa found in this study are widely reported in yellow baboons from elsewhere [36, 37, 39, 40] as well as in closely related olive and chacma (*P. ursinus*) baboons [29, 35]. In *Papio*, gastrointestinal ciliates are typically identified as *Balantioides coli*, however, genetic sequencing of microscopically indistinguishable ciliates, obtained from multiple NHP species, suggests numerous species of *Balantioides*-like and *Buxtonella*-like ciliates inhabit NHP gastrointestinal tracts [59]. Therefore, we report *Balantioides*-like ciliates as 'unidentified isotrichid ciliates'. One noticeable difference was the absence of *Trichuris* in Issa samples, a parasite widely documented in baboons [29, 36–40], though also absent from savannah chimpanzees (*P. troglodytes schweinfurthii*) surveyed in the Issa Valley region [41]. As *Trichuris* is a soil-transmitted helminth (STH), the hot and dry conditions of western Tanzania's dry season may be unsuitable for the *Trichuris* life cycle, with a low probability of survival during the soil living stage [41]. However, other STH (*Strongyloides* and strongylids) were present, despite requiring a moist external environment for transmission [41]. Examination of all sympatric NHPs would be required to determine if *Trichuris* was absent from specific primate taxa or the entire region, only then yielding further explanation.

In addition, while strongylids are commonly reported amongst baboons, our reported 100% prevalence is high compared to previous studies, where prevalence ranged from 5.9% to 75.4% [10, 38, 40].

Association with human disturbance

Prevalence of some parasite taxa varied with disturbance level, with most showing decreased prevalence with increased habitat disturbance. Gastrointestinal protozoans and metazoans have previously shown richness reduction in association with habitat degradation in other NHPs: black howler monkeys (*Aloutta pigra*) [60] and Udzungwa red colobus (*Procolobus gordonorum*) [10], although these observations were not mirrored in yellow baboons inhabiting the same area [10]. A reduction in either parasite richness or prevalence could be expected if gastrointestinal protozoans and metazoans respond similarly to human disturbance as many of their animal hosts do, with decreasing population size and diversity [10]. Intuitively, if host numbers, range or densities decrease, the same may be expected for their parasites due to reduced transmission rates [60]. Additionally, conversion of natural habitat for human land use often alters environmental conditions, reducing prevalence of parasite taxa with poor resilience to environmental change [60]. Counterintuitively, reduced gastrointestinal parasite diversity, associated with human disturbance, may be of great concern for wildlife hosts, with many gastrointestinal protozoans and metazoans now considered as vital gut symbionts. These gastrointestinal communities are now believed to be important for maintaining host health and fitness, calling for further research and its inclusion in conservation strategies as a method of endangered species health monitoring [10].

Contrasting findings may be explained by some parasite taxa being more susceptible to the environmental pressures of human disturbance. Convolutions may also arise through microscopic methodologies being unable to reveal all taxa to species or genus level, meaning parasite richness is often a combination of species richness and higher-level taxonomic richness. This calls into question the reliability of parasite richness as a measure for evaluating NHP gastrointestinal parasite communities, with some single parasite taxa in fact representing multiple species.

Besides parasite prevalence, we also evaluated the abundance of strongylids and unidentified isotrichid ciliates according to human disturbance, using EPGs and CPGs respectively. It is debated if intensity of parasite infection is linearly correlated with eggs/cysts shed in faeces, due to temporal variation of egg/cyst output [61–63]. However, with various studies identifying a linear relationship between egg output and adult worm burden within the host [64–66], and a lack of data for wild baboons, we assume egg/cyst output still provides a reliable insight into parasitic infections, as done previously in parasitic research of both *Papio* [39] and other NHP hosts [67–70].

Strongylid nematodes showed no association with level of human disturbance, however, increasing abundances of isotrichid ciliates significantly associated with increased human disturbance. While parasite abundances are less well documented than prevalence data, previous research has shown increases in abundance of other parasite taxa (*Strongyloides* and *E.histolytica*) with increases in human disturbance [17, 71]. Human disturbance could potentially increase parasite abundance indirectly through increased chronic stress, with high cortisol ('the stress hormone') levels previously correlated with increased parasite susceptibility in NHPs [18]. Alternatively, previous research found *B. coli* (an isotrichid ciliate) abundance was higher amongst crop-raiding baboons than wild foraging baboons [29]. While no systematic dietary observations were made here, all troops in the high disturbance area were within one kilometre of cropland and some were seen crop-raiding. Those in the low disturbance area were not in any proximity to cropland. Individuals observed crop raiding were consuming cassava, a highly starch rich crop [72]. Diets high in starch based carbohydrates have been shown to increase *B. coli* growth within hosts [73], therefore the increased abundance of isotrichid ciliates in the high disturbance area might be explained by starch rich diets resulting from

crop-raiding. While habitat disturbance, due to decreased resource availability, may reduce host nutritional status and thus increase parasite susceptibility in some NHPs [74], this is unlikely to be the case in baboons. *Papio* are typically described as dietary generalists [75], showing adaptability of foraging techniques to anthropogenic food sources [76]. As such, foraging in disturbed areas may in fact be energetically beneficial to *Papio* hosts [77], with anthropogenic food, such as crops, providing nutrient and energy-rich dietary supplementation [60], highlighting the potential impact of increased starch intake as previously mentioned.

Impact of age and group size

We found group size to be associated with prevalence of some parasite taxa, mostly protozoans, which results from increased animal density and exposure risk amongst larger groups [78]. While sociality, the tendency to form social living groups, has many benefits for wild NHPs [79], increased risk of parasite infection is one well-documented cost [80, 81], with spatial cohesion and contact between individual hosts facilitating parasite transmission [82, 83]. Larger group size may also be associated with higher parasite prevalence due to increased resource competition between group members and social stress amongst host individuals [33, 84] leading to poor nutrition and thus increased parasite susceptibility.

While we found no relationship between age and parasite abundance, we found baboon age to be associated with prevalence of two parasite taxa (*Strongyloides* sp. and *E. coli*). Age has previously been linked to parasite prevalence, abundance, and richness in baboons [35, 39], although the direction of these relationships varies between parasite taxa. Increased prevalence in juveniles, as we observed, may be explained by increased susceptibility to parasite infection, less active parasite avoidance or increased exposure risk resulting from age based behavioural differences [35]. Additionally, juveniles may lack fully developed immune systems [85], increasing their susceptibility to parasites [86], while older individuals may acquire immunity to certain parasite stages through repeated exposure [87].

Conservation implications

Some parasite taxa (*Strongyloides*, *Blastocystis*, *Entamoeba*, isotrichid ciliates and strongylids) found in our samples could have zoonotic potential. However, we highlight that we did not examine inter-specific parasite transmission in this study. *Balantioides coli*, an isotrichid ciliate, is both non-typical and often asymptomatic in humans, though reported in rural areas of some countries [88]. In some cases, *B. coli* infection of humans results in diarrhoea with potential for colonic invasion, while rare cases report more serious effects of inflammatory infections and pulmonary haemorrhage [88]. Likewise, *Strongyloides* is also reported in humans and can be asymptomatic [89]. Mixed infections, of multiple *Strongyloides* species, have been reported in both humans and NHPs [25, 90], suggesting zoonotic potential. *Blastocystis* is believed to have larger zoonotic potential, resulting from low host specificity [91]. While *Blastocystis* is commonly reported as part of a healthy human gastrointestinal biome [92], it is also linked to diarrhoea, vomiting and bloating [93]. While *Entamoeba* infection reported in humans is typically of different species to those reported in NHPs, human:NHP shared species have been identified, flagging the zoonotic potential of *Entamoeba* [94]. The symptoms of *Entamoeba* infection are again varied but can cause amebiasis, a potentially fatal intestinal illness [95]. The zoonotic potential of the other parasite taxa found during this study is less understood, although there is still potential for human:NHP transmission [96]. Consequently, shifting patterns of zoonotic parasite infections in wild hosts, resulting from human disturbance, could have implications also for the epidemiology of human parasite infections, heightening the value of considering parasitology within endangered species management.

Conclusions

Yellow baboons of the Tongwe Forest Reserve exhibit parasite fauna similar to other baboons across Tanzania. Factors influencing both parasite prevalence and abundance varied amongst parasite taxa, providing insight into the complexity of a host's parasite fauna.

Although we hypothesized that parasite prevalence would increase with human disturbance level, we found the opposite trend, with some taxa showing reduced prevalence in the highly disturbed area. This may result from symbiont organisms showing similar responses to habitat disturbance and degradation as many host species. On the other hand, high levels of human disturbance predicted increased isotrichid ciliate abundance, potentially explained by starch rich diets of baboons in the disturbed area. In future, inclusion of nutritional ecology assays would determine dietary shifts [97] associated with human disturbance, potentially linking with changes in gastrointestinal communities. While measurement of glucocorticoids in faecal samples could provide valuable insight into host stress and fitness [98] and attempt to explain how human disturbance may cause observed differences in gastrointestinal parasite communities.

Further research focused on parasite epidemiology and human:NHP transmission, including sampling of humans and livestock inhabiting the same environment, would be useful in determining the zoonotic parasite risk ongoing habitat disturbance poses to humans. In addition, gastrointestinal communities should be considered as a health monitoring method within NHP conservation strategies, particularly when addressing the threat of human disturbance.

Supporting information

S1 Table. Association of age, group size and habitat disturbance with parasite prevalence. Table showing all statistical results assessing the association between gastrointestinal parasite prevalence in yellow baboons and estimated age, group size and level of habitat disturbance.
(XLSX)

S2 Table. Gastrointestinal parasite infections of yellow baboons in western Tanzania. Table showing raw data on gastrointestinal parasite infections of 135 individuals, assessed through coproscopic analysis of faecal samples.
(XLSX)

S3 Table. Human disturbance scores of yellow baboon habitat in western Tanzania. Table showing transect scores of transects assessing the human disturbance of natural habitat within the home range of nine yellow baboon troops in western Tanzania.
(XLSX)

Acknowledgments

We thank the Tanzanian Wildlife Research Institute (TAWIRI) and Commission for Science and Technology (COSTECH) for permission to conduct research in Tanzania, as well as the Tanganyka and Uvinza District Governments. We also thank the field assistants of Greater Mahale Ecosystem Research and Conservation (GMERC) for invaluable help with data collection. The UCSD/Salk Center for Academic Research and Training in Anthropogeny (CARTA) provides long-term support to GMERC. We are enormously grateful to constructive comments from two anonymous reviewers on previous versions of this manuscript.

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Shutt-Phillips, K., Pafčo, B., Heistermann, M., Kasim, A., Petrželková, K.J., Profousová-Pšenková, I., Modrý, D., Todd, A., Fuh, T., Dicky, J.F., Bopalanzognako, J.B., Setchell, J.M.

2021

Fecal glucocorticoids and gastrointestinal parasite infections in wild western lowland gorillas (*Gorilla gorilla gorilla*) involved in ecotourism.

Gen. Comp. Endocrinol. 312, 113859. https://doi.org/10.1016/j.ygcen.2021.113859

General and Comparative Endocrinology 312 (2021) 113859

Contents lists available at ScienceDirect

General and Comparative Endocrinology

journal homepage: www.elsevier.com/locate/ygcen

Research paper

Fecal glucocorticoids and gastrointestinal parasite infections in wild western lowland gorillas (*Gorilla gorilla gorilla*) involved in ecotourism

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ARTICLE INFO

Keywords:
Primate
Conservation
Endocrine
Parasites
Immunity

ABSTRACT

Wildlife ecotourism can offer a source of revenue which benefits local development and conservation simultaneously. However, habituation of wildlife for ecotourism can cause long-term elevation of glucocorticoid hormones, which may suppress immune function and increase an animal's vulnerability to disease. We have previously shown that western lowland gorillas (*Gorilla gorilla gorilla*) undergoing habituation in Dzanga-Sangha Protected Areas, Central African Republic, have higher fecal glucocorticoid metabolite (FGCM) levels than both habituated and unhabituated gorillas. Here, we tested the relationship between FGCM levels and strongylid infections in the same gorillas. If high FGCM levels suppress the immune system, we predicted that FGCM levels will be positively associated with strongylid egg counts and that gorillas undergoing habituation will have the highest strongylid egg counts, relative to both habituated and unhabituated gorillas. We collected fecal samples over 12 months in two habituated gorilla groups, one group undergoing habituation and completely unhabituated gorillas. We established FGCM levels and fecal egg counts of *Necator/Oesophagostomum* spp. and *Mammomonogamus* sp. Controlling for seasonal variation and age-sex category in strongylid infections we found no significant relationship between FGCMs and *Nectator/Oesophagostomum* spp. or *Mammomonogamus* sp. egg counts in a within group comparison in either a habituated group or a group undergoing habituation. However, across groups, egg counts of *Nectator/Oesophagostomum* spp. were lowest in unhabituated animals and highest in the group undergoing habituation, matching the differences in FGCM levels among these gorilla groups. Our findings partially support the hypothesis that elevated glucocorticoids reduce a host's ability to control the extent of parasitic infections, and show the importance of non-invasive monitoring of endocrine function and parasite infection in individuals exposed to human pressure including habituation process and ecotourism.

8.20

Petrželková, K.J., Uwamahoro, C., Pafčo, B., Červená, B., Samaš, P., Mudakikwa, A., Muvunyi, R., Uwingeli, P., Gilardi, K., Nziza, J., Noheri, J.B., Eckardt, W., Ndagijimana, F., Ssebide, B., Okwirokello, R., Nizeyimana, F., Kambale Syaluha, E., Nzayisenga, G., Flores Girón, L., Bahizi, M., Ntwari, A.E., Lukusa, J.-P., Tumushime, J.C., Mangura, D., Mapilanga, J., Kalonji, A., Aruho, R., Stryková, A., Tehlarová, Z., Cameira, R., Lowenstine, L., Šlapeta, J., Romportl, D., Ferrari, N., Cranfield, M., Modrý, D.

2021

Heterogeneity in patterns of helminth infections across populations of mountain gorillas (*Gorilla beringei beringei*).

Sci. Rep. 11, 10869. https://doi.org/10.1038/s41598-021-89283-4

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Heterogeneity in patterns of helminth infections across populations of mountain gorillas (*Gorilla beringei beringei*)

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Conservation efforts have led to the recovery of the endangered mountain gorilla populations. Due to their limited potential for spatial expansion, population densities increased, which may alter the epidemiology of infectious diseases. Recently, clinical gastrointestinal illnesses linked to helminth infections have been recorded in both gorilla populations. To understand drivers and patterns of helminth infections we quantified strongylid and tapeworm infections across both Virunga Massif and Bwindi populations using fecal egg counts. We assessed the impact of age, sex, group size, season and spatial differences used as a proxy, which reflects observed variation in the occurrence of gastrointestinal problems, vegetation types, gorilla subpopulation growth and associated social structure on helminth infections. We revealed striking geographic differences in strongylid infections with higher egg counts mostly in areas with high occurrences of gastrointestinal disease. Increased helminth egg counts were also associated with decreasing group size in some areas. Observed spatial differences may reflect mutual effects of variations in subpopulation growth rates, gorilla social structure, and vegetation associated with altitude across mountain gorilla habitat. Helminth infection intensities in Virunga gorillas were lowest in the youngest and the oldest animals. Elucidating parasite infection patterns of endangered species with low genetic diversity is crucial for their conservation management.

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Endangered mountain gorillas (*Gorilla beringei beringei*) live in two populations and entirely within protected areas: one ranging within the Virunga Massif (VM) spanning the borders of Rwanda, Uganda and the Democratic Republic of the Congo (DRC), and the second ranging in the Bwindi-Sarambwe ecosystem in Uganda and DRC. The VM population declined dramatically in 1970s[1], but the numbers stabilized in the 1980s, and since then the population has been steadily increasing with annual growth rates around 3–4% between 2003 and 2016[2,3]. However, this increase has been almost entirely attributed to the growth rate in habituated groups[2–4] due to the "extreme conservation" measures these groups benefit from, such as daily group monitoring and protection, veterinary interventions and anti-poaching patrols[5]. Moreover, the population increase was not observed uniformly across VM, which may be due to varying ecological conditions that are linked to different habitat types[3,4,6,7]. We have less information about the history of the Bwindi-Sarambwe population dynamics. Robbins et al.[8] previously indicated that this second mountain gorilla population showed little or no growth before 2009 in comparison to VM population, but subsequent censuses in 2011 and 2018 detected growth of the population[9,10].

Although both mountain gorilla populations are growing, their potential spatial expansion is extremely limited due to high human densities adjacent to protected areas inhabited by mountain gorillas. Consequently, increasing numbers of gorillas in both populations are likely to result in rising individual and group densities at least in some areas. The VM population has already exceeded the previously predicted carrying capacity of the environment[11], as defined by the maximum number of animals an area will support based on resources[9]. In addition, between Mt. Karisimbi and Mt. Visoke (aka Bisoke) in the VM, where some of the highest growth rates of gorilla numbers occurred[3,4], the mountain gorillas experienced major changes in the social structure leading locally to a threefold increase in group densities. Namely, three stable multi-male groups, which have been steadily growing from the mid-1990s until 2006, underwent a series of group fissions and formation of smaller groups including multiple one-male groups[12,13]. As a result, there has been an increase in home range overlap, a reduction in home range areas used exclusively by a single group and annual intergroup encounter rates tripled in this area[13]. In addition, small groups, which are often one-male groups, may experience higher stress levels than large groups, which are often multi-male groups, because they have higher home range overlaps with neighboring groups, inter-group encounter rates, and are more vulnerable to infanticide by external males[14–17]. All those observed changes in population dynamics, social structure, and habitat use may be altering stress levels and cause changes in pathogen epidemiology with subsequent health problems[12,18,19].

Parasites influence the dynamics of free-living animal populations[20,21] through their impact on host survival[22] or reproduction[4]. The severity of parasite-induced diseases often depends on the intensity of infection (number of parasite individuals in the host animal)[23]. Identification of factors affecting parasite infections and their intensities as well as resulting patterns of parasite infections across host populations is essential for optimizing effectiveness of parasite control strategies and management of wildlife, particularly for species of conservation concerns[24]. For soil-transmitted parasites, host population density and habitat use play a central role in the host-parasite interaction by increasing the probability that parasite infectious stages will be ingested by a host[25]. Data from 6670 individual hosts representing 19 mammalian species showed a strong positive correlation between the host's population density and average gastrointestinal (GIT) strongylid nematode infection intensity[26]. In non-human primates this phenomenon has been demonstrated in fragmented populations, where host densities may increase due to restricted available habitat (e.g.[27,28]). In addition, host habitat characteristics can alter the risk of helminth infections[29]. For example, plant species composition, the leaf form of plants[30], temperature, and humidity[31,32] are known to influence helminth larval abundance, development and their migratory behavior[29]. Moreover, it is generally accepted that parasite abundance decreases with elevation[33], but it is not always true across all parasite taxa. For example, the survival of *Cooperia* and *Ostertagia* strongylids in livestock is optimized for cooler weather due to larval inhibition and thus the infection risk increases at higher elevations[34]. Seasonal differences in helminths (especially strongylid nematodes) are commonly observed in non-human primates (e.g.[35–38]). However, some studies reported highest infection intensities in the wet, while others in the dry season. The impact of host factors, such as sex and age, on parasite infections has also been well documented in non-human primates (e.g.[38,39]). It is widely accepted that males of vertebrate hosts tend to exhibit higher rates of parasitism and disease than females[40–42], with several main factors claimed to cause sex differences in parasitism: body size dimorphism with possibly larger nutritional requirements in the larger sex, social relationship, diet, habitat and hormones[43]. Three theoretical models are generally used to describe the host age–infection relationship in parasites; Type I (linear) and Type II (asymptotic) relationships are thought to occur in the absence of an effective host immune response and instead depend on immigration-death processes linked to parasite transmission and longevity[44]. Type III (convex) associations have been suggested to indicate adaptive immunity to parasites[45] and have been reported in non-human primates[36].

Gastrointestinal helminthiases are typically asymptomatic in wild non-human primates, but host factors like immune status, or extrinsic factors, such as habitat loss, fragmentation and other anthropogenic pressures, can alter the transmission dynamics of helminths and increase host susceptibility, which may exacerbate negative effects of infections on health[46,47]. However, clinical diseases caused by parasites have been observed in mountain gorillas; post-mortem examinations of 60 gorillas from 1985 to 2007 (Mountain Gorilla Veterinary Project [MGVP], publication in review) revealed histopathologic evidence for enteritis in more than 50% of cases, gastritis in more than 35% of cases, and colitis in more than 25% of cases. More recently (2013–2017), several cases of severe gastritis have been diagnosed in VM in Rwanda, particularly in adult gorillas ranging between Mt. Karisimbi and Mt. Visoke, with most cases involving young silverbacks (MGVP, data on file). Trichostrongylid nematodes have been observed in the stomach and small intestines in these cases and the histopathology of observed stomach lesions are typical of those caused by nematodes in a range of domestic and wild mammals, including non-human primates[48–50]. In Bwindi Impenetrable National Park (BINP), the Ugandan part of the Bwindi-Sarambwe ecosystem, similar alarming observations indicating change in parasitic infections have been recorded (MGVP, data on file). Many gorillas have exhibited weight loss, declining body condition and poor

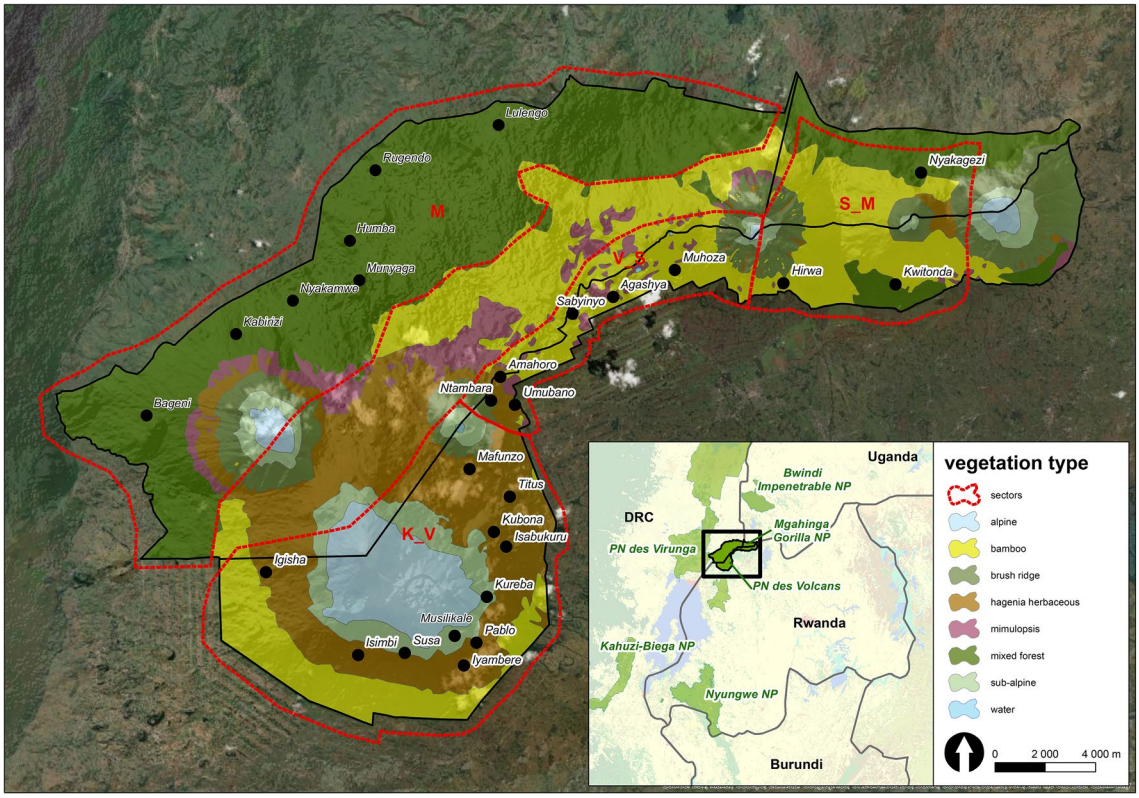


Figure 1. Division of the Virunga Massif into four areas according to volcano positions (M: Mikeno, Virunga National Park; K_V: Karisimbi–Visoke, Volcanoes National Park; V_S: Visoke–Sabyinyo, Volcanoes National Park; S_M: Sabyinyo–Muhavura, Mgahinga Gorilla National Park, Volcanoes National Park) with vegetation zones (based on Robbins et al.[5]) and distribution of the studied groups. Map was created using ArcGIS Desktop 10.6 (ESRI 2019. ArcGIS Desktop: Release 10.6. Redlands, CA: Environmental Systems Research Institute; esri.com). Vegetation data were adopted according to Robbins et al.[5]; boundaries of protected areas were derived from ProtectedPlanet.net database.

haircoats in recent years, and in severe cases (n > 10) anthelmintic treatment were administered to sick gorillas in BINP resulting in marked clinical improvement. Anoplocephalid tapeworms have also been commonly observed during necropsies in gorillas from both VM and BINP, and intensities of infection have been high in some animals (e.g., > 100 tapeworms in one animal) (MGVP, data on file).

Although parasites of mountain gorillas have been studied for almost 100 years[51], surveys of the spatio-temporal pattern of parasite infections over the entire geographic distribution of mountain gorillas are lacking (for basic studies covering gorilla subpopulations see e.g. [38,52–54]). We conducted an extensive species-wide survey of helminth infections across the VM and BINP populations and across different seasons to uncover the drivers and patterns of helminth infections and provide a comprehensive foundation for future assessments of the impact of these parasites on gorilla population dynamics. We utilized samples collected from night nests across both populations and samples from individually-identified gorillas from five social groups in VM in Rwanda that experienced differing levels of strongylid infections based on a small-scale pilot study using the same methods applied in this larger-scale study. We divided the VM into four areas according to volcano positions (modification of Weber and Vedder[1], Fig. 1). This geographic division allowed for a comparison of areas with differences in the occurrence of parasite-related GIT disease, as well as differences in vegetation types, and in historical subpopulation growth and associated current social structure across mountain gorilla distribution range[55,56]. The areas were namely: Karisimbi–Visoke (K_V), Visoke–Sabyinyo (V_S), Sabyinyo–Muhavura (aka Muhabura) (S_M), Mikeno (M). The entirety of BINP was considered as one area. We then investigated the impact of area, sampling period (season), group size and host factors (sex, age) on helminth infections.

In the samples collected from the night nests, we predict that the helminth infections (quantified as eggs per gram in fecal samples; hereafter EPG or egg counts) are higher in the areas with a high occurrence of GIT problems (K_V) than in areas where less or no GIT problems were reported (M, S_M). We predicted that the helminth infections are higher in silverback gorillas in comparison to other age/sex classes and higher in smaller gorilla groups than in larger groups as smaller groups may experience more stress. We further predicted that these effects of age/sex class and group size on the helminth infections are stronger in the areas with higher occurrence of GIT problems and historically higher subpopulation growth. In individually sampled gorillas we predicted that the helminth infections are higher in males than in females, change with age following the convex pattern, and differ between groups as our small-scale pilot project involving five groups suggested. We predicted that age and sex effects are more pronounced in groups with higher helminth egg counts and that convex pattern of egg counts

Park	Area (Weber & Vedder, 1983)[1]	Sampling period (2018)	Season	Sample type
VoNP, Rwanda	Karisimbi–Visoke (K_V) Visoke–Sabyinyo (V_S) Sabyinyo–Muhavura (S_M)	January/February May/June September/October	Dry Boundary wet/dry Wet	Nest Individual Nest + individual
ViNP, DRC	Mikeno (M)	January/February September/October	Dry Wet	Nest Nest
MGNP, Uganda	Sabyinyo–Muhavura (S_M)	September/October	Wet	Nest
BINP, Uganda	BINP	September/October	Wet	Nest

Table 1. Sampling design by area (with a national park and country), sampling period and its corresponding season and sample type (for more detailed information about sampled groups, see the Supplementary Table S1). *VoNP* Volcanoes National Park in Rwanda, *ViNP* Virunga National Park in Democratic Republic of the Congo, *MGNP* Mgahinga Gorilla National Park in Uganda, *BINP* Bwindi Impenetrable National Park in Uganda.

is more pronounced in males than females. In both night nest and individual samples we expect differences in helminth infections between sampling periods, which correspond with different seasons and predicted higher egg counts in the wet season[38].

Results

We used three different datasets to test our hypotheses aiming to explain drivers and patterns of helminth infections across five subpopulations (K_V, V_S, S_M, M, BINP) of the mountain gorillas (Fig. 1, Table 1 and Table S1, for details see "Material and methods"). Dataset 1 included nest samples collected across the whole VM and BINP during a single period (n = 392) and was used to investigate the effects of area, age/sex class and group size. Dataset 2 was composed of nest samples but restricted to VM covering two periods (January/February and September/October 2018) (n = 491) and used to examine the effect of area, age/sex class, group size and sampling period (season). Dataset 3 with samples from individually identified gorillas from selected groups in VM collected across two periods (May/June 2018 and September/October 2018) (n = 450) was used to investigate the effects of group identity, sex, exact age[2] and sampling period (season).

We observed helminth eggs in the majority of examined gorilla fecal samples (Fig. 2): in dataset 1, the prevalence of the strongylids was 97% (median EPG = 480; range 0–10,050), while the prevalence of the tapeworms was 88% (median EPG = 188; range 0–5775). For the information on strongylid and tapeworm infections in datasets 2 and 3 see Table S1.

Strongylid infections. In dataset 1, we detected an interactive effect of area and age/sex class (Wald's Chi-square = 26.7, df = 12, p = 0.008; Table 2) with no clear pattern in M and S_M areas, while in other areas the infants had lower EPG values than non-infant classes (Fig. 3A). M and S_M areas had similar EPG values (Wald's Chi-square = 1.7, df = 1, p = 0.19) and both had lower EPG values than the other areas (Wald's Chi-square test, all $p < 0.001$; Fig. 3A, Table S1). Area K_V had lower EPG values than V_S and BINP areas (Wald's Chi-square test, both $p < 0.01$), while the V_S and BINP had similar infection rates (Wald's Chi-square = 0.1, df = 1, p = 0.92). Infants had lower EPG values than any other age/sex classes (Wald's Chi-square test, all $p < 0.01$), while no differences were observed among other sex/age classes (Fig. 3A, Table S1).

In dataset 2, we found an interactive effect of area and group size (Wald's Chi-square = 20.0, df = 3, $p < 0.001$; Table 3). The EPG values decreased with increasing group size in the S_M area (estimate = -0.14 ± 0.03; z = −5.0, $p < 0.001$) but there was no relationship in other areas (Fig. 4A). All the areas differed from each other (Wald's Chi-square test, all $p < 0.04$; Fig. 4A,B, Table 3). EPG values were lower in infants compared to other age/sex classes (Wald's Chi-square test, all $p < 0.002$), while no differences were observed among other age/sex classes (Fig. 4B, Table S1).

In dataset 3, EPG values differed among groups (Wald's Chi-square = 323.9, df = 4, $p < 0.001$; Table 4) with Kwitonda group (from S_M sector) showing lower EPG values compared to any other group (Wald's Chi-square test, all $p < 0.001$), while the other groups had similar values (Fig. 5A, Table S1). There was a quadratic age effect (Wald's Chi-square = 6.8, df = 2, p = 0.03) on strongylid EPG values with a peak occurring between 10 and 30 years (Fig. 5A). Gorillas showed higher EPG values in September/October (wet season) (estimated marginal mean ± SE = 645 ± 197 EPG) compared to May/June (boundary wet/dry season) (429 ± 130; Wald's Chi-square = 41.3, df = 1, $p < 0.001$; Fig. 5B).

Tapeworm infections. In dataset 1, there was interactive effect of area with both group size (Wald's Chi-square = 10.8, df = 4, p = 0.03) and age/sex class (Wald's Chi-square = 32.1, df = 12, p = 0.001) on tapeworm EPG values (Table 2, Fig. 3B,C). Specifically, the EPG values decreased with increasing group size in K_V (estimate = -0.11 ± 0.03; z = −4.3, $p < 0.001$) and M area (estimate = -0.08 ± 0.03; z = −2.3, p = 0.02; Fig. 3B) but no relationship was found in other areas. Differences in EPG values between areas were detected only between K_V and BINP (Wald's Chi-square = 10.0, df = 1, p = 0.002), with higher values in BINP. There was a significant interaction of area and age/sex on EPG values, where K_V area showed a non-significant tendency to have lower EPG values in infants than in other age/sex classes while other areas had rather similar EPG values across age/sex classes (Fig. 3C).



Figure 2. Examined host and their parasites quantified in the study: (**A**) resting group of mountain gorillas in Virunga Massif (Virunga National Park, Democratic Republic of the Congo); (**B**) egg of strongylid nematode; (**C**) egg of anoplocephalid tapeworm; both microphotographs in the same scale.

Effect	*d.f.*	Strongylids		Tapeworms	
		Chi_square	*P value*	*Chi_square*	*P value*
Area	4	264.6	< 0.001	19.4	< 0.001
Age/sex	3	33.9	< 0.001	8.4	0.04
Group size	1	0.01	0.91	14.4	< 0.001
Area: age/sex	12	26.7	0.008	32.1	0.001
Area: group size	4	2.7	0.61	10.8	0.03

Table 2. Factors influencing helminth infections in Virunga Massif and Bwindi Impenetrable National Park (dataset 1: nest samples from all national parks).

In dataset 2, we found an interactive effect of area and group size (Wald's Chi-square = 14.0, df = 3, p = 0.003; Table 3, Fig. 4C). The EPG values decreased with increasing group size in the K_V area (estimate = −0.12 ± 0.02; z = −5.2, p < 0.001) but no changes in EPG values with group size were observed in other areas (Fig. 4C). EPG values in K_V area differed significantly from S_M (Wald's Chi-square = 5.1, df = 1, p = 0.02) and V_S areas (Wald's Chi-square = 6.5, df = 1, p = 0.01; Fig. 4C, Table S1). Similar to strongylid EPG values, infants had lower EPG values for tapeworms compared to any other age/sex classes (Wald's Chi-square test, all p < 0.01), while the egg counts between other classes were similar (Fig. 4D, Table S1).

In dataset 3, there was an interactive effect of quadratic age and group identity on EPG values (Wald's Chi-square = 40.9, df = 8, p < 0.001; Table 4). In particular, EPG values peaked between the age of 10 and 20 years in groups Titus, Hirwa and Kureba with the highest peak found in Titus group (Fig. 5C, Tables 4 and S1). Kwitonda group had the lowest maximum EPG value with the latest peak (age = 26 years). Individuals had higher EPG

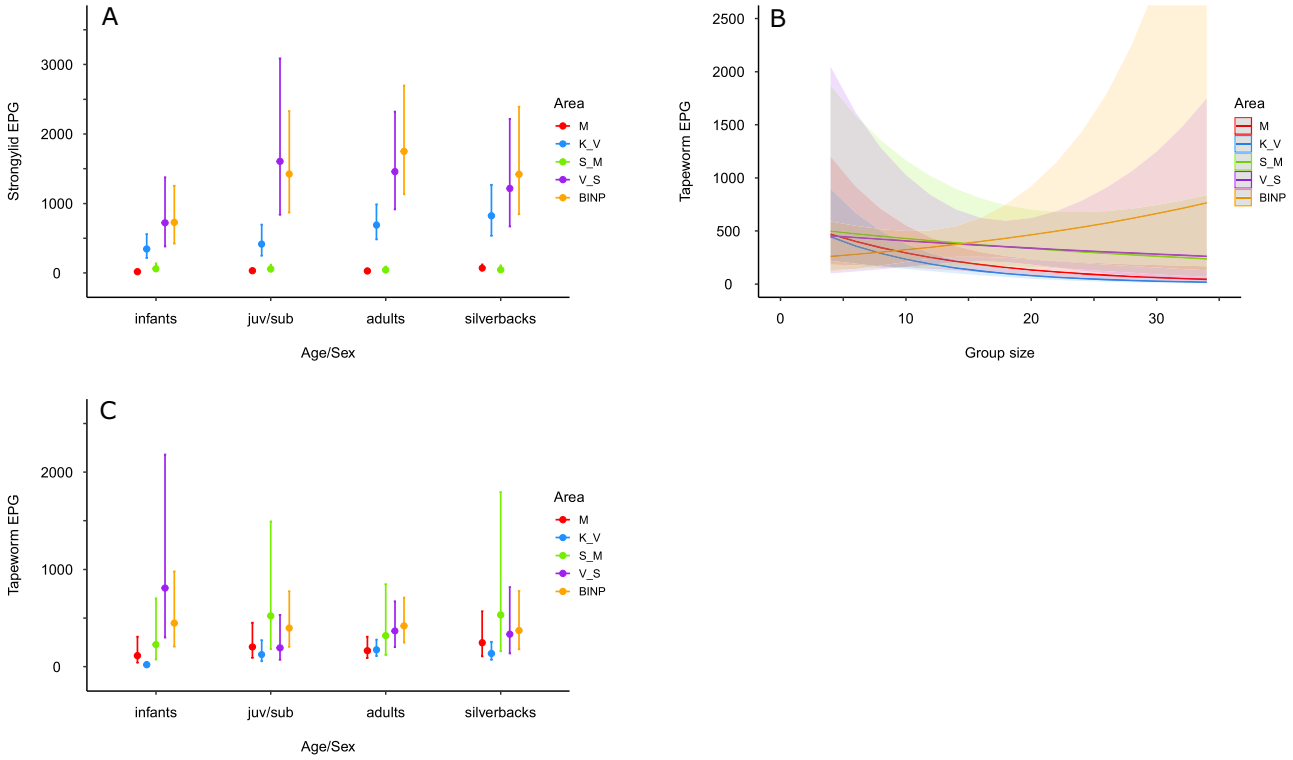


Figure 3. Models' predicted mean values with 95% confidence intervals for the factors affecting helminth infections in mountain gorillas in Virunga Massif and Bwindi Impenetrable National Park (dataset 1). (**A**) strongylid eggs per gram (EPG)—sampling area and age/sex class effects, (**B**) tapeworm EPG—sampling area and group size effects, (**C**) tapeworm EPG—sampling area and age/sex class effects. juv/sub: juvenile/subadult class; M: Mikeno (Virunga National Park); K_V: Karisimbi–Visoke (Volcanoes National Park), V_S: Visoke–Sabyinyo (Volcanoes National Park), S_M: Sabyinyo–Muhavura (Mgahinga Gorilla National Park, Volcanoes National Park).

		Strongylids		Tapeworms	
Effect	***d.f.***	***Chi_square***	***P value***	***Chi_square***	***P value***
Area	3	376.3	< 0.001	8.6	0.03
Age/sex	3	41.6	< 0.001	21.6	< 0.001
Group size	1	6.1	0.01	15.2	< 0.001
Season	1	0.04	0.85	0.07	0.79
Area: age/sex	9	15.0	0.09	15.2	0.09
Area: group size	3	20.0	< 0.001	14.0	0.003

Table 3. Factors influencing helminth infections in Virunga Massif (dataset 2: nest samples from Volcanos and Virunga National Parks from wet and dry season).

values in September/October (wet season) (estimated marginal mean ± SE = 625 ± 97 EPG) compared to May/June (boundary wet/dry season) (361 ± 97 EPG; Wald's Chi-square = 37.6, df = 1, p < 0.001; Fig. 5D).

Discussion

The majority of mountain gorilla fecal samples examined were positive for both strongylid nematodes and anoplocephalid tapeworms, which is in accordance with previous studies of mountain gorilla gastrointestinal (GIT) parasites[38,52,53,57]. The overall highest strongylid egg counts were recorded in the gorilla groups in the BINP, where individual cases of weight loss and poor body condition have been documented, followed by the groups from the Visoke–Sabyinyo area (V_S) of the VM. Gorilla groups ranging in the Karisimbi–Visoke area (K_V), where the highest number of severe gastritis in the VM have been documented, had notably high strongylid EPG values, especially when compared with the groups ranging in the Mikeno (M in in DRC) and the Sabyinyo–Muhavura (S_M) area of the VM, which exhibited substantially lower egg counts and no fatal GIT diseases in recent years (MGVP, unpublished data). Strongylid egg counts increased with decreasing group size in the S_M area (without

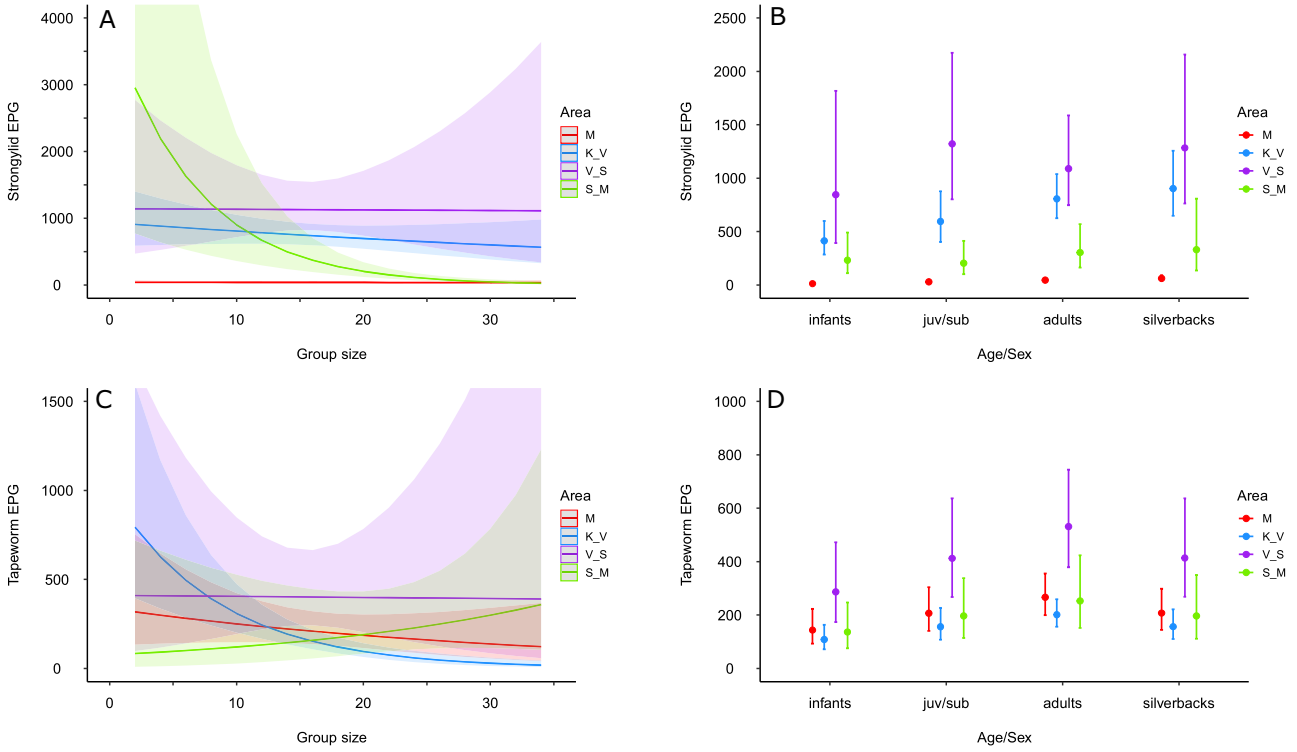


Figure 4. Models' predicted mean values with 95% confidence intervals for the factors affecting helminth infections in mountain gorillas in Virunga Massif in two periods (dataset 2). (**A**) strongylid egg per gram (EPG)—sampling area and group size effects, (**B**) strongylid EPG—sampling area and age/sex class effects, (**C**) tapeworm EPG—sampling area and group size effects, (**D**) tapeworm EPG—sampling area and age/sex class effects. juv/sub: juvenile/subadult class; M: Mikeno (Virunga National Park); K_V: Karisimbi–Visoke (Volcanoes National Park), V_S: Visoke–Sabyiniyo (Volcanoes National Park), S_M: Sabyinyo–Muhavura (Volcanoes National Park).

Effect	*d.f.*	Strongylids		Tapeworms	
		Chi_square	*P value*	*Chi_square*	*P value*
Age^2	2	6.8	0.03	9.2	0.01
Group ID	4	323.9	< 0.001	16.8	0.002
Season	1	41.3	< 0.001	29.6	< 0.001
Sex	1	1.8	0.18	0.8	0.38
Age^2: group ID	8	11.7	0.17	40.9	< 0.001
Age^2: sex	2	2.4	0.31	3.1	0.21
Group ID: sex	4	0.4	0.98	7.5	0.11

Table 4. Factors influencing helminth infections in the individual samples from five selected groups from Volcanos National Park collected in wet and dry season.

GIT problems) and we did not observe higher helminth infections in silverbacks compared to other age/sex classes, which does not support our predictions. On the other hand, according to our prediction tapeworm infection intensities increased with decreasing group size in an area with high occurrence of GIT problems (K_V) but also in an area with no GIT problem (M). The observed differences in helminth infection rates among sampling areas thus only partially reflect occurrence of detected GIT problems in mountain gorillas.

Mutual effects of different population growth of VM mountain gorilla subpopulations and the BINP population, differences in the social structure particularly in VM areas, as well as differences in habitat characteristics (vegetation types interlinked with altitude) across the distribution range of mountain gorillas may explain observed differences in strongylid infections among areas. Unfortunately, information about current gorilla densities across the sampled areas are lacking, but different population dynamics have been observed in the VM areas with low strongylid egg counts (see M and S_M in Fig. 4A) in comparison to the other VM areas. For

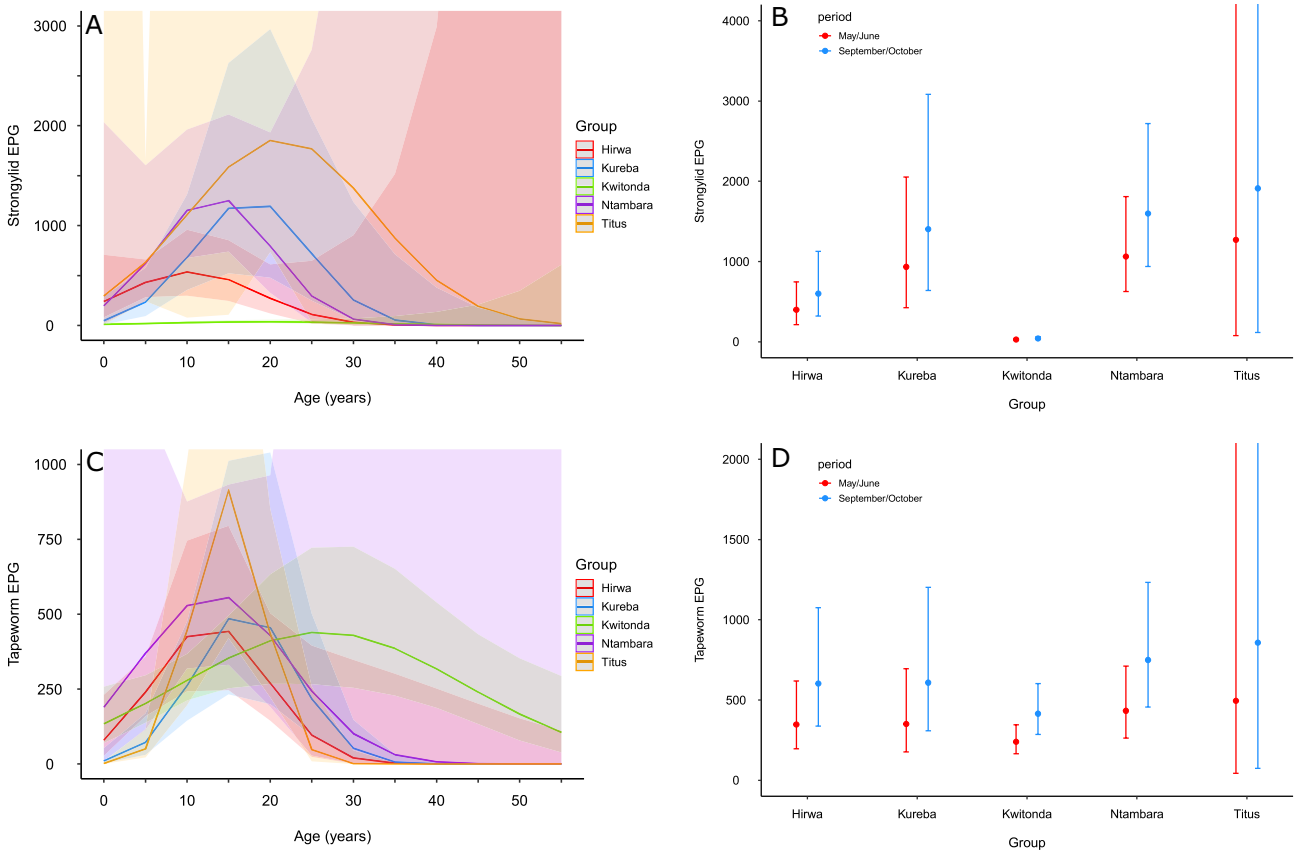


Figure 5. Models' predicted mean values with 95% confidence intervals for the factors affecting helminth infections in individually sampled mountain gorillas in Virunga Massif (dataset 3). (**A**) strongylid eggs per gram (EPG)—group identity and age effects, (**B**) strongylid EPG—sampling period (season) and group identity effects, (**C**) tapeworm EPG—group identity and age effects, (**D**) tapeworm EPG—sampling period (season) and group identity effects.

example, the VM population decline between 1960 and 1973 was driven by a severe decrease in the M (Mikeno) subpopulation in DRC (67%), accounting for 85% of the total VM decline, while in the other VM areas the population decline was much lower[1]. Since the 1980s the number of gorillas in M either declined again[7] or did not significantly increase[4,6]. Considerably less gorillas have been found historically in S_M than in other areas of the VM and the size of this subpopulation has been stable for years[1,3,4,6,7]. In contrast, in the central areas of VM where strongylid egg counts were high (e.g. in K_V and V_S), the increase in gorilla numbers and groups since the 1980s has been the most significant[1,3,4,7,11].

Our results thus indirectly suggest that high growth rates of gorilla subpopulations in specific VM areas in the last 40 years can be linked to high strongylid infection intensities found in these areas today. Existing data from BINP does not allow comparisons of population changes between VM and BINP, but although differences in strongylid egg counts among groups in BINP were observed (see Supplementary Table S1), we did not detect any groups with apparent low EPG, as was observed in some areas of VM (M and S_M areas). However, groups from the northern BINP area, which is characterized by lower gorilla and group densities as well as medium-altitude moist evergreen forest[9], were not included in this study and thus should be investigated in future sampling efforts. Comparisons of our results with previous studies conducted in BINP are difficult due to significant differences in employed quantification methods. However, egg counts reported in previous studies were also high, exceeding 1000 and reaching values up to 11,000 EPG even in formalin-fixed fecal samples, where lower egg counts are always observed due to fixation[38,53,54]. Comparison of strongylid egg counts between both mountain gorilla populations and conclusions on observed high strongylid egg counts in BINP should be done with caution, as BINP is characterized by different vegetation linked to different environmental factors, presence of different sympatric non-human primates[9] and thus the structure of the strongylid communities could differ between both gorilla populations. The very high egg counts in BINP groups and observed clinical cases of chronic weight loss and declining body condition, which may be attributable at least in part to helminth infection intensities warrant further and more detailed research of helminth infections across BINP including precise strongylid identification. In general, further studies with detailed information about current gorilla and group densities across the whole VM and BINP are urgently needed to fully understand the ability of helminths, in particular strongylids, to regulate the growth of mountain gorilla populations.

Observed relatively high strongylid EPG values and the increase of tapeworm EPG values with decreasing group size in K_V may be associated with the disintegration of a few larger multi-male groups after 2006, which led to high group densities, a higher proportion of one-male groups and groups below average size in this area[12,13]. One-male groups, which are often smaller in size, are more vulnerable to attacks from external males, which can cause lethal wounds in adult males and infants[16,17]. Therefore, higher home range overlaps, the higher rates of interaction between groups in K_V, which can be extremely stressful events[18], may have compromised the immune system of the gorillas, in particular the small and one-male groups, leading to an increased disease susceptibility, probable higher parasite infection intensities, and subsequently to impairing health[58]. Gorilla social structure has not been studied in detail in other areas, however census results suggest a gradual increase in the number of gorilla groups in V_S (with the highest strongylid egg counts) area since 2000[3,4,6,7], which may have resulted in similar conditions as documented in K_V[13] leading to high strongylid EPG values.

Marked differences in the vegetation cover (changing with elevation) that dominate gorilla habitat in our studied VM areas may also in part explain observed strongylid EPG differences among these areas. The gorilla groups with lower strongylid EPG values in the M and the S_M area predominantly utilize lower altitude forest (mixed forest) habitat, while the groups with higher strongylid EPG values in K_V and V_M exclusively range in vegetation zones at higher altitudes, including the bamboo zones, *Hagenia – Hypericum*, and subalpine[5,59]. Moreover, the recent increase in group densities in K_V[12] also caused more groups in this area to routinely range at extremely high elevations above 3300 m (Dian Fossey Gorilla Fund—DFGF, unpublished data). In addition, the strongylid infections could be affected by gorilla diet, particularly by the consumption of medicinal plants[60], which may vary in their availability across the VM vegetation zones. Very little is known about self-medication or even on diet composition in less studied VM areas (M, S_M). Comprehensive studies on the survival of strongylid larvae in different vegetation/elevation zones in VM are needed to better understand the impact of factors interlinked to vegetation cover on infection intensities. We cannot rule out that VM gorillas living in the lower altitude mixed forest with higher tree densities and more food in trees[11] are more arboreal than those ranging in the other higher vegetation zones with lower tree densities, which may reduce their contact with infective larvae and result in lower strongylid intensities.

In our study, the strongylid infection intensities were lower only in infants compared to other broad age/sex classes[3]. Silverbacks, which have been diagnosed more frequently with fatal gastritis, did not exhibit the highest strongylid infections as predicted. The dataset composed of samples from individually-identified gorillas revealed no differences in helminth infections between males and females and that helminth infections in VM gorillas exhibited a convex (Type III) age-parasite intensity profile[21], with the peak of infections around the age of 10–20 years for tapeworms and 10–30 years for strongylids. The youngest and the oldest animals had the lowest egg counts. There are several mechanisms that might account for convex age-intensity curves, including parasite-induced host mortality, acquired immunity, age-related changes in susceptibility to infection and age-dependent changes in exposure to parasites[39]. If acquired immunity influences helminth infections, than a negative correlation between infection level peak of infection and the age at which the peak occurs is expected—a phenomenon known as the 'peak shift' (see[23,61]). However, we observed different trends in the relationship of age and helminth egg counts and we suggest that other factors may be the drivers of the age-related helminth infection dynamics in VM gorillas. Namely, parasite-induced host mortality may be responsible for the convex (Type III) age-helminth intensity profile in mountain gorillas, which should be a focus for further study. Studies have shown that host-acquired immunity seems to be less effective at controlling strongylid infections[62,63], while in equids age-related exposure to tapeworm infection may be an important determinant of acquired immunity and up-to-date results do not preclude the development of acquired immunity[64].

Seasonal effects on both helminth infections were observed only when comparing helminth egg counts from May/June to those from September/October in individually sampled gorillas. We found that both strongylid and tapeworm EPG values were higher in September/October (wet season), which is in accordance with many studies in non-human primates as strongylid larvae have better conditions for survival and development during the wet season (e.g.[35,38]). In anaplocephalid tapeworms, epidemiological studies demonstrated significant seasonality with high infections linked to months with high humidity and moderate temperatures[65].

Extreme conservation efforts have led to the recovery of the mountain gorilla, but our study points to new challenges emerging as possible "side effects" of this remarkable conservation success. Unraveling the patterns of parasite infections in a population, evaluating host exposure to infective parasite stages and susceptibility to infection and its consequences on host health is important for the conservation and survival of endangered animal species[21,66]. Small, isolated populations with low genetic diversity such as the mountain gorillas[67] are particularly sensitive to pathogens as heterozygosity levels are linked directly to reduced population fitness via inbreeding depression[68]. The growing gorilla population has limited spatial expansion opportunities and those areas will be reaching their carrying capacity. Our study highlights that any results originating from the groups in K_V, which have been intensively studied since 1967, and from which most of our existing knowledge of the Virunga gorilla population stems, may not be applicable to the whole VM population and thus research efforts should be further expanded to groups across the VM including those ranging in the Democratic Republic of the Congo (M area). It is important to mention that strongylid nematodes in large terrestrial herbivores, such as great apes, occur in complex communities and identification of species within those communities using traditional coproscopic or even conventional molecular methods is not possible. Therefore, a strain-level identification of strongylids infecting gorillas using a metabarcoding approach is urgently warranted[69], as the gorillas from the areas with increased incidence of GIT disease may host different communities of strongylids in comparison to other areas.

Material and methods

Study sites. The study was carried out across the entire range of the mountain gorilla including: (1) the Virunga Massif (VM, 451 km^2), which spans three national parks—Volcanoes National Park (VoNP) in northwestern Rwanda, the Mikeno sector of the Virunga National Park (ViNP) in the North Kivu Province, eastern DRC and Mgahinga Gorilla National Park (MGNP) in southwestern Uganda; and (2) Bwindi Impenetrable National Park (BINP, 331 km^2) located in southwestern Uganda. Sarambwe Reserve, a small protected area in DRC that is contiguous with BINP, was excluded for logistical reasons (Fig. 1). There are two rainy seasons (March–May and September–November) and two dry seasons (December–February and June–August) in the study region. VM is characterized by an altitude ranging from 2000 to 4500 m[70], with different vegetation types mainly determined by elevation, including the alpine zone (above 3600 m), sub-alpine zone (3200–3600 m), *Hagenia–Hypericum* zone (2800–3200 m), lower altitude forest (mixed forest) (1600–2500 m), bamboo zone (2500–2800 m), disturbed woodland (2300–2800 m), open grassland and swamp (occurring in various altitudes)[55] (Fig. 1). The most abundant vegetation type in ViNP is the mixed forest, while in VoNP it is bamboo, followed by *Hagenia—Hypericum* woodland[55]. Historically, most of the mixed forest in VoNP was destroyed by human activities, especially in the 1950s and 1960s, which pushed the gorilla population on the Rwandan side to higher elevations, where temperatures can drop to 0 °C. A single small section of lower altitude forest remains between Mt. Gahinga and Sabyinyo (the S_M). MGNP is dominated by mixed forest and bamboo[55] (Fig. 1). The vegetation in BINP is classified as medium-altitude moist evergreen forest and high-altitude submontane forest. The altitude in BINP ranges from 1160 to 2607 m[56].

Sample collection. In 2018 fresh fecal samples were collected from habituated mountain gorillas across the VM and BINP (without northern part). Study groups in Bwindi predominantly ranged in the high-altitude submontane forest. In the morning, feces were collected from gorilla nests of the previous night, put into plastic bags, labeled with age/sex class (silverback > 12 years, adult > 8 years, subadult/juvenile > 3.5–8 years, infant 0–3.5 years) based on the estimation of the size of fecal lobes[3,71]. Groups from ViNP and VoNP (VM) were sampled twice, once in the dry season (January/February) and once in the wet season (September/October), to assess the impact of season (sampling period) on helminth infection intensities. To investigate the impact of age and sex as host factors more accurately than estimating age/sex classes from nest samples, individual samples were also collected during focal follows in five habituated gorilla groups in VoNP in VM in May/June and in September/October (Table 1).

The groups were assigned to particular areas in VM (see above for division) based on 2018 GPS points recorded daily by staff of MGVP, Rwanda Development Board (RDB) and DFGF in Rwanda and by ViNP and MGVP in DRC (Table 1; Table S1; Fig. 5). Data on group size (number of individuals), exact age, and sex of individually sampled gorillas were obtained from DFGF, MGVP, VoNP/RDB, ViNP, MGNP, BINP and Uganda Wildlife Authority databases. Fecal samples were transported to the MGVP laboratories in Musanze (Rwanda), Kisoro (Uganda) and Goma (DRC), stored in a refrigerator, and examined within a maximum of 48 h from estimated defecation.

Parasite quantification. The number of parasite eggs shed in feces (egg counts or eggs per gram; EPG) can be used as a proxy for nematode infection intensities in both domestic and wild animals, including non-human primates (e.g.[72,73]). Although the numbers may be influenced by individual and temporal changes in egg shedding[74,75], the relationship between egg counts and the number of adult worms in the host has been repeatedly confirmed[25,26]. In anoplolocephalid tapeworms this relationship can be more complicated; terminated gravid proglottids detach from an adult and can either disintegrate along their route leading to the release of eggs in feces, or proglottids are shed intact. However, based on studies in horses[76,77], we also implemented egg counts as a proxy for the quantification of tapeworm infections in gorillas.

From the inside of each fecal bolus we subsampled 3 g of feces, homogenized it in water, strained the suspended feces through a sieve into a 50 ml tube and centrifuged the mixture at 2500 rpm for 5 min. After decanting the supernatant, we quantified the strongylid and tapeworm eggs in the remaining sediment using the Mini-FLOTAC device[78]. Specifically, we resuspended the sediment in a saturated salt solution (NaCl, specific gravity = 1.2) to a total volume of 45 ml and filled both chambers of the Mini-FLOTAC using disposable Pasteur pipettes. After 10 min, the Mini-FLOTAC was read under a light microscope using 10× magnification. We counted all tapeworm and strongylid eggs and calculated eggs per gram of feces (EPG) using the following formula: EPG = total egg count in two chambers × 7.5.

Statistical analyses. We investigated factors influencing helminth infection intensities using univariate generalized linear mixed models (GLMMs) with strongylid and tapeworm fecal EPGs as response variables. With respect to research aims, we analyzed the EPGs in three different datasets: (1) dataset 1 (n = 392); (2) dataset 2 (n = 491); and (3) dataset 3 (n = 450) (Table S1).

In datasets 1 and 2, sampling area (categorical), group size (continuous; number of individuals) and age/sex class (categorical; "infants", "juvenile/subadults", "adults", "silverbacks") were considered as explanatory variables. In dataset 2, period (binary; season: "January/February" and "September/October") was included as an additional explanatory variable. We also included the interaction of sampling area with age/sex class or group size, respectively in both dataset 1 and 2. We could not rule out repeated sampling of an individual when using nest samples because a gorilla may build and use more than one nest per night[79]. Therefore, we included age/sex class nested within group identity as random effect in all models using datasets 1 and 2 to minimize the risk of Type I error. Therefore, the probability of considering the same sample as independent was lower[80]. In dataset 3, period (season), group identity (categorical), sex (binary) and age (continuous; in years) were considered

as explanatory variables. To include possible quadratic effects of age on helminth egg counts, its second-order polynomial effect was included. We further included interactions of group identity with polynomial age or sex, and the interaction of polynomial age with sex. GLMMs applied on the dataset 3 included individual identity as a random factor to account for the non-independence of repeated samples collected from the same individual.

We applied a negative binomial distribution to all models to account for the aggregated distribution of parasites within-host populations. Models were fitted with the function glmer.nb of the package "lme4" (version 1.1-23[81]) in R 3.5.0[82]. We validated each model fit using the variance inflation factor to assess multicollinearity, visual inspection of the model residuals, homoscedasticity of each variable residuals, random effect quantiles Q–Q plots and found them satisfactory. We presented and interpreted results from the full model including all the variables and their interactions or polynomials for which we made predictions in the introduction. This model approach allowed us to test our hypotheses and reduce Type I error and problems of biased effect sizes[83]. However, main effects are generally not biologically interpretable if involved in interactions or polynomial terms[84]. Thus, to enable interpretation of main effects in the presence of interactions or polynomials, we refitted each model with manually re-coded categorical variables (i.e. constructing dummy variables) and then centered (subtracted the sample mean from all the variable values) all the independent variables entering the full model (e.g.[85,86]). Significance tests for fixed effects were performed using Type II Wald's Chi-square tests under which we compared a focal dummy variable against remaining dummy variables. This was accomplished by setting the focal dummy variables as the reference (e.g. "infant" and "M" dummy variables were assigned with value 0) in the model structure. To compare/test all the relevant combinations, we re-fitted the model with a slightly modified model structure where we only changed the reference dummy variables. Albeit the full models allowed us to address predictions, we also wanted to identify biologically most relevant factors by finding model best fitting the data[87]. Thus, we further performed a complementary analysis to the best models using model selection procedure according to the Akaike information criterion corrected for small sample sizes (AIC_c)[88] with R package MuMIn[89]. Best models and their outputs were presented in the Supplementary material.

Ethics approval. This research involved only non-invasive work with wild non-human primates. All work was done in accordance with guidelines of the national authorities.

Data availability

Data are available in the Figshare repository https://figshare.com/s/61461cfcb8d9657c3bcb.

Received: 7 October 2020; Accepted: 20 April 2021
Published online: 25 May 2021

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Acknowledgements

We are thankful to the Rwanda Development Board, the Uganda Wildlife Authority, the Uganda National Council of Science and Technology and Institut Congolais pour la Conservation de la Nature for their long-term support and for the permission to conduct this research. We are indebted to the field staff of the Dian Fossey Gorilla Fund, Rwanda Development Board, Virunga National Park, Bwindi Impenetrable National Park and Mgahinga Gorilla National Park for their tireless support in collecting fecal samples. The study was supported by the Czech Science Foundation, Grant no. 18–24345S and the U.S. Fish and Wildlife Service Division of International Affairs, Grant no. F17AP00964.

Author contributions

K.J.P., B.P., B.Č., W.E., L.L., M.C. and D.M. designed the study. K.J.P., D.M. and M.C. obtained funding. C.U., B.P., B.Č, D.M., A.S., Z.T., R.C., J.Š., M.B. and D.M. examined the samples. N.F. and P.S. conducted statistical analyses and D.R. performed GIS analyses and prepared Fig. 1. R.M., A.M., P.U., K.G., J.N., J.B.N., W.E., F.N., B.S., R.O., F.N., E.K.S., G.N., L.F.G., M.B., A.E.N., J.-P.L., J.C.T., J.M., R.A. and A.K. managed the extensive sample collection across three national parks in three countries. K.J.P. wrote the first draft of manuscript. B.P. and B.Č. significantly improved the first version of the manuscript. K.G. and W.E. actively contributed to the manuscript. All co-authors read and approved the final version of the manuscript.

Competing interests

The authors declare no competing interests.

Additional information

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1038/s41598-021-89283-4.

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Petrželková, K.J., Samaš, P., Romportl, D., Uwamahoro, C., Červená, B., Pafčo, B., Prokopová, T., Cameira, R., Granjon, A.C., Shapiro, A., Bahizi, M., Nziza, J., Noheri, J.B., Syaluha, E.K., Eckardt, W., Ndagijimana, F., Šlapeta, J., Modrý, D., Gilardi, K., Muvunyi, R., Uwingeli, P., Mudakikwa, A., Mapilanga, J., Kalonji, A., Hickey, J.R., Cranfield, M.

2022

Ecological drivers of helminth infection patterns in the Virunga Massif mountain gorilla population.

Int. J. Parasitol. Parasites Wildl. 17, 174–184. https://doi.org/10.1016/j.ijppaw.2022.01.007

International Journal for Parasitology: Parasites and Wildlife 17 (2022) 174–184

Contents lists available at ScienceDirect

International Journal for Parasitology: Parasites and Wildlife

journal homepage: www.elsevier.com/locate/ijppaw

Ecological drivers of helminth infection patterns in the Virunga Massif mountain gorilla population

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ARTICLE INFO

Keywords:
Mountain gorilla
Helminth infection
Strongylid nematode
Tapeworm
Environmental and host factors

ABSTRACT

The Virunga Massif mountain gorilla population has been periodically monitored since the early 1970s, with gradually increasing effort. The population declined drastically in the 1970s, but the numbers stabilized in the 1980s. Since then, the population has been steadily increasing within their limited habitat fragment that is surrounded by a dense human population. We examined fecal samples collected during the Virunga 2015–2016 surveys in monitored and unmonitored gorilla groups and quantified strongylid and tapeworm infections using egg counts per gram to determine environmental and host factors that shape these helminth infections. We showed that higher strongylid infections were present in gorilla groups with smaller size of the 500-m buffered minimum-convex polygon (MCP) of detected nest sites per gorilla group, but in higher gorilla densities and inhabiting vegetation types occurring at higher elevations with higher precipitation and lower temperatures. On the contrary, the impact of monitoring (habituation) was minor, detected in tapeworms and only when in the interaction with environmental variables and MCP area. Our results suggest that the Virunga mountain gorilla population may be partially regulated by strongylid nematodes at higher gorilla densities. New health challenges are probably emerging among mountain gorillas because of the success of conservation efforts, as manifested by significant increases in gorilla numbers in recent decades, but few possibilities for the population expansion due to limited amounts of habitat.

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https://doi.org/10.1016/j.ijppaw.2022.01.007
Received 8 November 2021; Received in revised form 7 January 2022; Accepted 17 January 2022
Available online 20 January 2022
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1. Introduction

Mountain gorillas (*Gorilla beringei beringei*) inhabit two isolated forests: the Virunga Massif that spans the borders of the Democratic Republic of the Congo (DRC), Rwanda and Uganda and the Bwindi–Sarambwe ecosystem (Bwindi) that spans the border between Uganda and the DRC. The Virunga gorilla population is one of the longest monitored of all great apes; it has been periodically surveyed since the early 1970s when a low point of 260–290 individuals was estimated (Harcourt and Groom, 1972; Weber and Vedder, 1983; Stewart et al., 2001; Gray et al., 2005, 2010, 2011, 2013; Hickey et al., 2019). The population stabilized in the 1980s, and since then it has been steadily increasing, with annual growth rates around 3–4% between 2003 and 2016, resulting in estimated 639–669 individuals in 2015 and 2016 (Gray et al., 2011; Granjon et al., 2020). Virunga gorillas have been monitored daily since the late 1960s, and today approximately 60% of gorillas are habituated to human presence for tourism and research (Hickey et al., 2019). Population increase has been almost entirely attributed to growth of the habituated groups (Gray et al., 2010, 2013; Granjon et al., 2020), which benefits from "extreme conservation" measures, such as daily monitoring and protection, veterinary interventions and anti-poaching patrols (Robbins et al., 2011). In addition to habituation status, differences in growth rates across the Virunga Massif may also be due to varying ecological conditions that are linked to different vegetation types (Kalpers et al., 2003; Gray et al., 2011, 2013; Hickey et al., 2019). For example, some of the highest population growth rates have occurred between Mounts Karisimbi and Visoke (aka Bisoke) in the Virunga Massif (Gray et al., 2013). As a result, this gorilla subpopulation has experienced significant changes in social structure leading to a localized threefold increase in group density (Caillaud et al., 2014, 2020).

The changes in population dynamics, social structure, and habitat use may be altering stress levels (Harcourt et al., 2001; Caillaud et al., 2014; Eckardt et al., 2019), which could lead to changes in infectious disease epidemiology with possible health consequences. In the Virunga Massif, clinical disease caused by helminths has been observed in mountain gorillas (Muhangi et al., 2021). Notably, several cases of severe gastritis were diagnosed in adult gorillas ranging between Mounts Karisimbi and Visoke after group density had significantly increased (Petrželková et al., 2021). Infection risk for soil-transmitted helminths increases with host density, especially for non-human primates that form cohesive social units, because the opportunities for transmission increase with increased potential for contact with infective stages (Harcourt et al., 2001; Eckardt et al., 2019; Muhangi et al., 2021; Petrželková et al., 2021). In contrast, having a larger home range can reduce the density of hosts, thereby decreasing density-dependent infection risk (Freeland, 1976; Nunn et al., 2003; Lindenfors et al., 2007; Bordes et al., 2009).

Mountain gorillas are among the largest herbivores (Ripple et al., 2015) and their gastrointestinal parasite fauna corresponds to that of other non-ruminant herbivores, with a dominance of entodiniomorphid ciliates, strongylid nematodes (representatives of superfamilies Trichostrongyloidea and Strongyloidea - Rothman and Bowman, 2003; Pafčo et al., unpublished data) and anoplocephalid tapeworms (referred to as *Anoplocephala gorillae* and *Bertiella* sp. - Rothman and Bowman, 2003; Doležalová et al., 2015). While several classical studies of parasites in mountain gorilla subpopulations have been conducted (Ashford et al., 1996; Sleeman et al., 2000; Kalema-Zikusoka et al., 2005; Rothman et al., 2008), only recently there was a range-wide survey of helminths conducted across the Virunga and Bwindi populations to better understand the drivers and patterns of helminth infections (Petrželková et al., 2021). This study assessed the effects of age, sex, group size, season and spatial differences on helminth infections (quantified as eggs per gram in fecal samples). The spatial differences between sampling areas were a proxy for the occurrence of gastrointestinal disease, vegetation types, gorilla subpopulation growth and associated changes in social structure. Striking geographic differences in strongylid infections across the Virunga Massif were revealed, with higher fecal egg counts corresponding to areas with more gastrointestinal disease. This correspondence may reflect concomitant effects of subpopulation growth rates connected with gorilla densities, gorilla social structure, and vegetation types associated with elevational gradients across the Virunga Massif. Increased helminth egg counts were also associated with decreasing group size in some areas across Virunga Massif (Petrželková et al., 2021).

Importantly, the study of Petrželková et al. (2021) was restricted to only habituated (monitored) gorillas, which limited the interpretation of the results regarding drivers of helminth infections across the entire Virunga gorilla population. Ape habituation is beneficial to both tourism, research and protection (Higham and Shelton, 2011; Robbins et al., 2011), but it may be stressful for the species concerned (Doran-Sheehy et al., 2007; Shutt et al., 2014). The chronic stressors associated with tourism and research could significantly reduce ape immunity, thereby increasing their susceptibility to diseases (Hofer and East, 1998; Woodford et al., 2002; Shutt et al., 2014). Although it has been demonstrated that habituation may not necessarily pose a greater risk of protist and helminth infections in western lowland gorilla groups (Pafčo et al., 2017), evaluation of the helminth infections intensities indicated increased strongylid intensities in a group under habituation with high fecal glucocorticoid metabolite levels (Shutt-Phillips et al., 2021).

In the Virunga Massif, unmonitored (unhabituated) gorilla groups are only sampled during intensive full-population surveys of each subpopulation that occur approximately every five years (Hickey et al., 2019). Here, we examined fecal samples collected during the Virunga Massif 2015–2016 surveys (Hickey et al., 2019) for strongylid and tapeworm infections (expressed as eggs per gram in fecal samples; hereafter EPG or egg counts) to determine the factors that may be influencing helminth infections in both habituated (monitored) and unhabituated (unmonitored) gorilla groups in the Virunga Massif. As well, because the impact of particular factors (e.g., vegetation types, growth in historical Virunga subpopulations, and associated current social structure) known to influence parasite infection was assessed only via spatial proxy in the previous publication (Petrželková et al., 2021), we evaluated the effects of a more detailed suite of host and environmental factors on helminth infections, namely the effect of geographic distance between groups, group size, size of the 500-m buffered minimum-convex polygons (MCP) of detected nest sites per gorilla group and elevation, annual precipitation, temperature, relative density of gorillas (each expressed as a mean per 500-m buffered MCP) and percent cover of select vegetation types within each 500-m buffered MCP.

2. Material and methods

2.1. Study site

We surveyed helminth infections in the portion of the Virunga Massif (416 km^2) comprising Volcanoes National Park in northwestern Rwanda and the Mikeno sector of Virunga National Park in eastern DRC (Fig. 1). The Virunga Massif is characterized by an elevation ranging from 1800 to 4500 m (McNeilage, 2001), with vegetation types mainly determined by elevation (Owiunji et al., 2005). The most abundant vegetation type in the Virunga National Park (DRC) is mixed forest, whereas in the Volcanos National Park (Rwanda) it is bamboo, followed by *Hagenia* forest and *Hypericum* woodland (Owiunji et al., 2005). Historically, human activities destroyed most of the mixed forest in the Volcanos National Park, thereby forcing the gorilla population to higher elevations, where temperatures can drop to 0 °C. A single small fragment of mixed forest in lower elevation remains between Mts. Gahinga and Sabyinyo in the Volcanos National Park (Fig. 1).

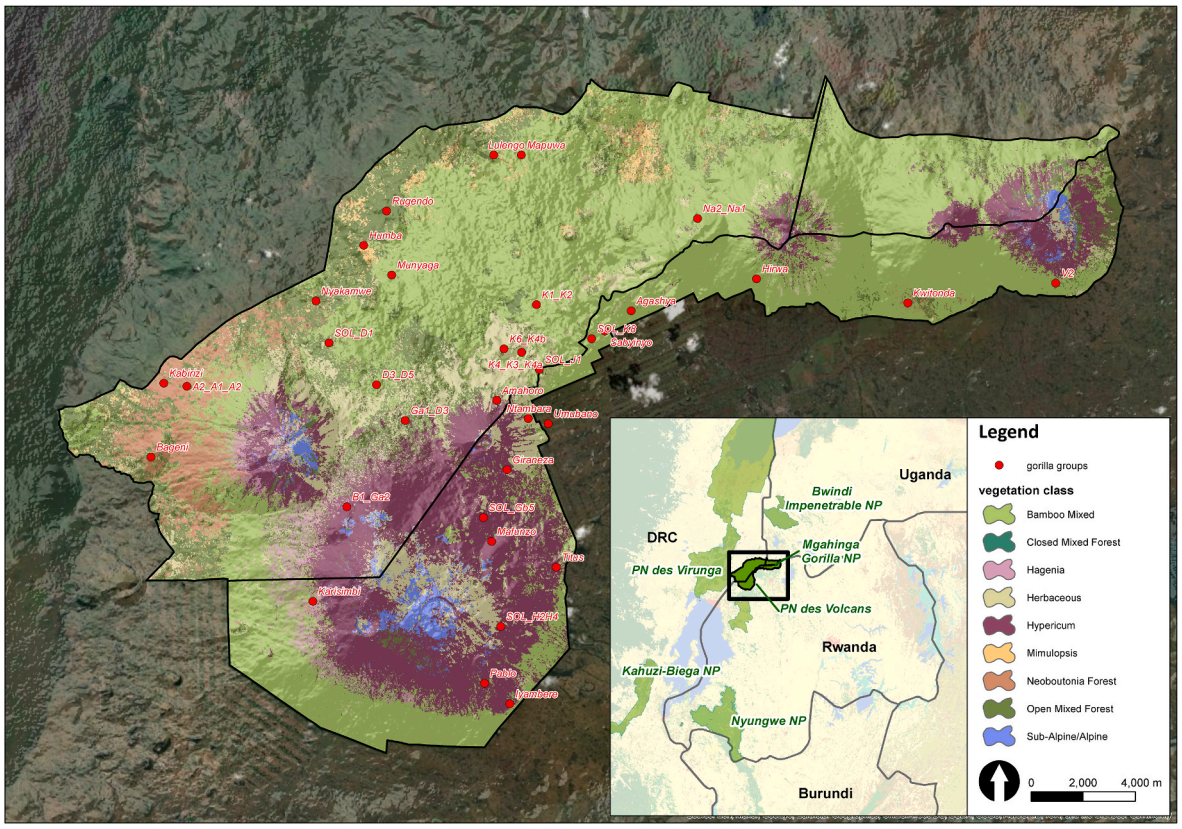


Fig. 1. Location of study gorilla groups during the Virunga Massif 2015–2016 surveys (Hickey et al., 2019) expressed as centroids of their 500-m buffered minimum-convex polygon. Vegetation data were adopted according to WWF-Germany and IGCP 2017; boundaries of protected areas were derived from ProtectedPlanet.net database. Map was created using ArcGIS Desktop 10.8 (ESRI, 2020. ArcGIS Desktop: Release 10.8. Redlands, CA: Environmental Systems Research Institute; esri.com).

2.2. Sample and data collection

Field survey methods for fecal collection from gorilla nest sites are described fully in Hickey et al. (2019) and Granjon et al. (2020). Briefly, teams of four or five trained census technicians walked through the forest without the aid of trails following approximate compass bearings. These reconnaissance routes were spaced approximately 500-m apart. Reconnaissance routes departed from the bearing and became irregular when teams maneuvered around geographic features, such as ravines or peaks, and when teams detected fresh gorilla signs. As they walked, teams listened and looked for signs – such as trails, vocal calls, tracks, and dung – of gorillas and selected large mammals.

At every location where gorilla or other mammal signs were recorded, teams also recorded the dominant vegetation type within a circle of 10-m radius around the observation (Hickey et al., 2019). Teams entered all data in Panasonic FZX1 Toughpad™ handheld devices, which provided highly accurate geographic coordinates for the observation sites. Dominant vegetation was categorized into the following vegetation types, roughly following the classification of Watts (1983), Plumptre (1991) and Grueter et al. (2013): pure bamboo, mixed bamboo, *Hagenia* forest, *Hypericum* woodland, herbaceous, meadow, *Mimulopsis*, closed mixed forest, open mixed forest, *Neobutonia* forest, subalpine and alpine. Geo-referenced locations of vegetation types collected during this survey were used as ground-truth data in a supervised landcover classification of Sentinel 2 satellite imagery (https://sentinel.esa.int/) (Fig. 1) (WWF-Germany and IGCP, 2017). That separate classification effort produced the vegetation map used for this study.

Upon encounter with a fresh gorilla trail, teams would leave the compass bearing and follow the trail in search of gorilla nest sites. At gorilla nest sites, teams collected samples of gorilla feces from each nest and preserved them in 90% ethanol for subsequent genetic identification of gorilla groups and individuals (Granjon et al., 2020). Additionally, from October to December 2015, teams also collected 3-g fecal samples (<1 day old) into tubes containing 10% formalin for helminth analysis; these samples were assigned to their corresponding individual and gorilla group based on aforementioned genetic analyses (Granjon et al., 2020). These formalin-fixed fecal samples collected in the first sweep were used for this study and only one sample per individual was examined.

2.3. Minimum convex polygon calculation

There were not enough nest-site locations per group (typically 1 to 6 points/group) to estimate home ranges per gorilla group; therefore, we instead calculated the minimum convex polygon (MCP) corresponding to each group using data from both sweeps. Each MCP equated to the smallest polygon that contained all the nest-site locations for a given group and in which no internal angle exceeded 180°. While MCPs do not represent home ranges, which are probably larger for this population of mountain gorillas, they provide a basis for relating the relative density of gorillas of a given group to a suite of covariates. To compensate for the small size of the MCP resulting from so few nest-site locations collected during this survey, we buffered each MCP by 500 m based on estimates that mountain gorillas move up to approximately 500 m daily (Grueter et al., 2013, 2018). Hereafter, we refer to 500-m buffered MCPs as simply 'MCPs'.

We estimated the relative density of gorillas in ArcGIS 10.6 (ESRI, 2020) across Virunga Massif excluding the Ugandan portion. First, we attributed the centroid of each gorilla group's MCP with the group size (number of gorillas), as determined by either genetics (unmonitored

groups) or daily counts (monitored groups) (Granjon et al., 2020). Using Spatial Analyst tools (ESRI, 2020), we calculated the kernel density (KD) across the study area based on these centroids and their corresponding group sizes. We set the search radius to 1 km. These steps provided a raster in which every pixel across the study area contained a value of relative density of gorillas.

2.4. Extraction of environmental variables

We calculated the mean relative density of gorillas per 500-m buffered MCP to characterize the relative density of gorillas experienced per group. Additionally for each MCP we calculated the: (i) mean elevation (SRTM USGS, 2020), (ii) mean annual temperature, (iii) mean annual precipitation and (iv) percentage of the following select vegetation types from the above definition used during the 2015–2016 survey: bamboo (pure or mixed), *Hagenia* forest, *Hypericum* woodland, herbaceous, closed mixed forest, open mixed forest, *Mimulopsis*, and *Neoboutonia* forest. We based both climate variables on WorldClim 2.0 (worldclim.org) 1-km resolution data verified by CRU (Climatic Research Unit; https://sites.uea.ac.uk/cru/data). We calculated Euclidean distances between centroids of all MCP to express proximity or level of isolation per gorilla group. All spatial data were harmonized and analyzed in ArcGIS Desktop 10.8 environment (ESRI, 2020), using standard geoprocessing tools (e.g., spatial analysis, zonal statistics, raster algebra).

2.5. Fecal sample analyses

We homogenized each fecal sample, strained it through a sieve, further diluted it with non-sterile tap water to a volume of 50 ml and centrifuged (5 min 2500 rpm). We weighed the sediment to allow for egg per gram feces counts (EPG), and then resuspended the sediment in 10% formalin to a total volume of 10 ml. We then centrifuged 1 ml of fecal suspension and examined the whole sediment from this 1 ml, while counting strongylid nematode and tapeworm eggs. The number of eggs per gram of sediment was calculated according to the following formula: $n = N \times 10/m$, where N = number of eggs in examined amount of sediment, and m = weight of examined sediment (Pafčo et al., 2017; Doležalová et al., 2018). The number of helminth eggs shed in feces (egg counts or eggs per gram; EPG) is used as a proxy for helminth infection intensities in both domestic and wild animals, including non-human primates and gorillas (see Petrželková et al., 2021 for detail description).

2.6. Statistical analyses

All the analyses were performed in R 4.3.0 (R Core Team, 2020). We employed a univariate generalized linear mixed model (GLMM) with negative binomial distribution separately for strongylid and tapeworm EPG as response variables to explore factors shaping helminth infections using the package glmmTMB [version 1.0.2.1; (Brooks et al., 2017)]. We detected substantial correlations among the environmental variables (Table 1). To overcome issues associated with the multicollinearity, we performed a principal component analysis by standardizing these 10 environmental variables and then projecting them onto principal components (PC) using the function 'prcomp'.

We selected the first two principal components (hereafter PC1 and PC2) explaining 44.3% and 17.6% of the total variation, respectively. PC1 correlated positively with mean annual precipitation, mean elevation of the MCP (area of 500-m buffered minimum convex polygon of detected nest sites per gorilla group; see *Minimum convex polygon calculation* for details) and % of *Hagenia* forest and *Hypericum* woodland in the MCP, and negatively with mean annual temperature of the MCP and % of open and closed mixed forests and *Neoboutonia* forest within the MCP (Table 2, Fig. 2). PC2 correlated positively with % of bamboo (pure and mixed) within the MCP and negatively with % of *Hypericum* woodland, *Neoboutonia* forest, and open and closed mixed forests (Table 2, Fig. 2).

We included effects of group size (count; number of individuals), monitoring (habituation) status (binary; monitored or unmonitored), MCP (continuous; area of 500-m buffered minimum convex polygon of detected nest sites per gorilla group), density (continuous; mean relative density of gorillas within the MCP) and PC1 and PC2 (continuous; principal components of the environmental factors) into the GLMMs. Both explanatory variables contained an excess of zeroes (27 and 23%); therefore, we first ran negative binomial GLMMs with and without zero-inflation correction and compared them using the function 'anova' in R. Results suggested that the GLMM corrected for zero-inflation fits the data significantly better for both strongylid EPG (AIC = 1718.2 vs. 1722.5; $p = 0.01$) and tapeworm EPG (AIC = 1932.5 vs. 1967.8; $p < 0.001$). We therefore used the GLMM corrected for zero-inflation in further analyses. We included a random effect of group identity (N = 35 groups) in all statistical models to account for repeated sampling within

Table 2
Correlations between original variables and the first two principal components (PC1 and PC2). Vegetation types were computed as % per groups' MCP (area of 500m buffered minimum convex polygon of detected nest sites per gorilla group; see Minimum convex polygon calculation for details); elevation, annual temperature and annual precipitation as means per groups' MCP.

Variable	PC1	PC2
Temperature	−0.41	0.02
Open mixed forest	−0.32	−0.26
Closed mixed forest	−0.26	−0.46
Neoboutonia forest	−0.25	−0.45
Bamboo (pure or mixed)	−0.14	0.64
Herbaceous	0.08	0.05
Hagenia forest	0.26	−0.17
Precipitation	0.36	−0.11
Hypericum woodland	0.40	−0.26
Elevation	0.47	−0.08

Table 1
Pearson's correlation matrix for seven vegetation types, two climatic variables and one geographical variable characterizing gorilla groups' MCPs (area of 500-m buffered minimum convex polygon of detected nest sites per gorilla group; see Minimum convex polygon calculation for details). Vegetation types were computed as % of each group's MCP; elevation, annual temperature and annual precipitation were expressed as means for each group's MCP. Bamboo vegetation included both pure and mixed types. 'f.' = forest, 'w.' = woodland.

	Bamboo	Closed mixed f.	*Hagenia* f.	Herbaceous	*Hypericum* w.	*Neoboutonia* f.	Open mixed f.	Precipitation	Temperature	Elevation
Bamboo	1	−0.20	−0.19	−0.32	−0.54	−0.28	−0.18	−0.30	0.27	−0.36
Closed mixed f.		1	−0.14	−0.21	−0.25	0.54	0.51	−0.29	0.43	−0.44
Hagenia f.			1	−0.15	0.32	−0.12	−0.25	0.41	−0.47	0.55
Herbaceous				1	−0.07	−0.22	−0.13	0.14	−0.08	0.13
Hypericum w.					1	−0.25	−0.44	0.64	−0.75	0.85
Neoboutonia f.						1	0.25	−0.31	0.46	−0.47
Open mixed f.							1	−0.49	0.52	−0.61
Precipitation								1	−0.39	0.75
Temperature									1	−0.88
Elevation										1

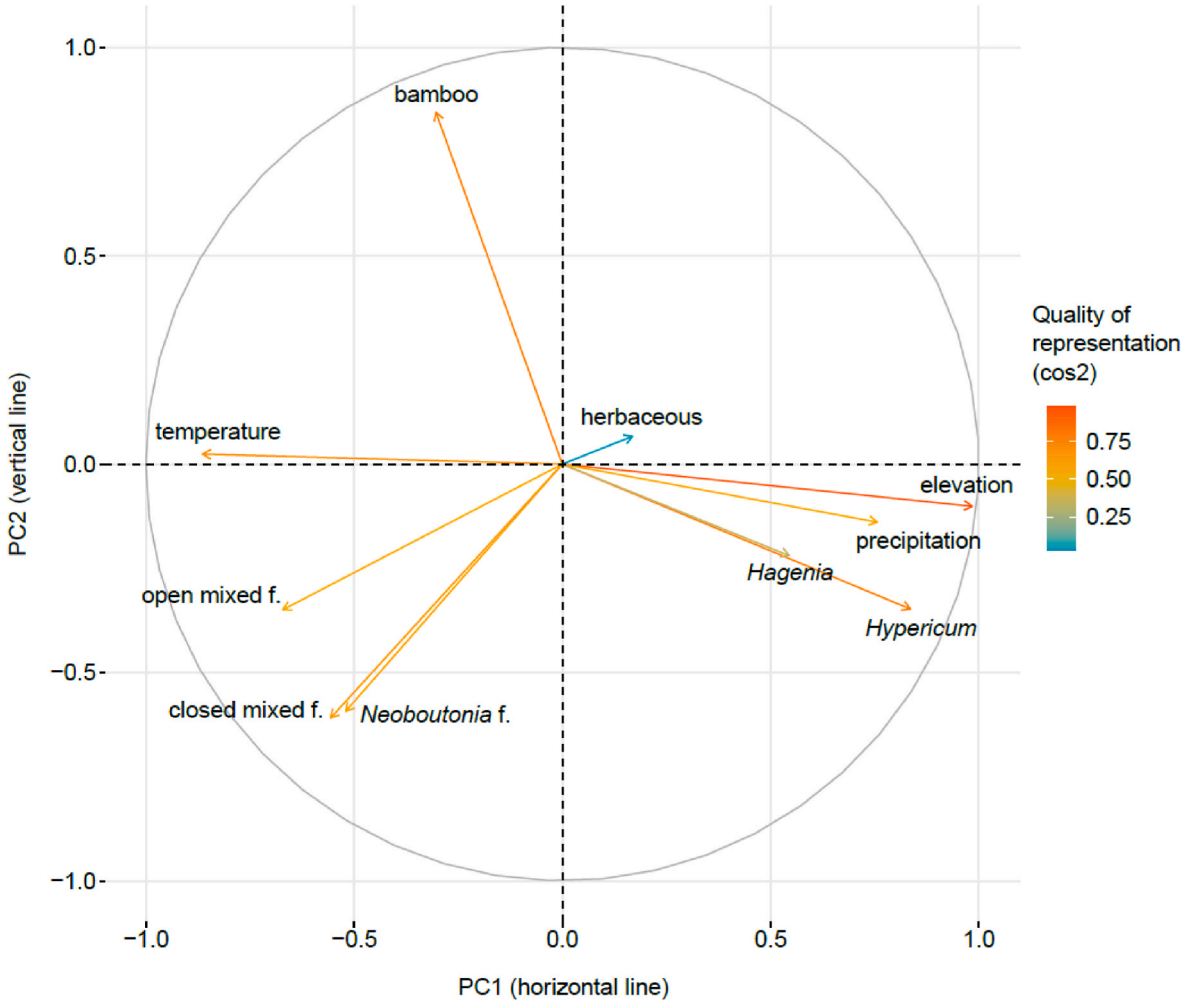


Fig. 2. Principal component analysis output showing associations between variables and the first two principal components PC1 and PC2. Each variable contribution to principal components and its quality are represented by length of vector and its color, respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

groups. We further examined a potential effect of geographical distance among gorilla groups by comparing full models with and without the Gaussian spatial correlation structure. For this purpose, we performed Moran's I test for spatial autocorrelation on the calculated quantile residuals using the function 'testSpatialAutocorrelation' from DHARMa package [version 0.3.3.0; (Hartig, 2020)]. This test showed that there was no significant spatial dependence among gorilla groups for both strongylid ($p = 0.16$) and tapeworm EPG ($p = 0.83$). In addition, visual inspection of variograms did not suggest spatial dependence. Moreover, GLMMs with spatial correlation structure produced identical estimates and conclusions as those without the structure. Thus, we used a zero-inflated negative binomial GLMM without spatial correlation structure in the analyses. We presented results from both the full model, including all the predictors and their interactions, and the minimal model. The minimal model was created by removing non-significant predictors from the best model, where the best model was selected from a suite of models comprised of all possible combinations of covariates from the global model according to Akaike Information Criterion corrected for small sample size (AICc) using the package MuMIn (version 1.43.17) (Barton, 2020). We interpreted only the result outputs of the minimal model.

Because GLMMs outputs cannot be reliably evaluated with standard residual plots, we employed recent diagnostic methods from the R package DHARMa and its goodness-of-fit test functions 'testDispersion', 'testUniformity' and 'testZeroinflation' (Hartig, 2020). Using this package, we obtained intuitively interpretable residuals by standardizing them to values between 0 and 1. This is achieved by a simulation-based approach, similar to the Bayesian p-value or the parametric bootstrap that transforms the residuals to a standardized scale. The generated standardized residuals did not suggest significant heterogeneity or deviations from the expected distribution and their overall distribution conformed to expectations (all $p > 0.05$). We further examined if our GLMMs sufficiently accounted for zero-inflation in response variables. Again, observed number of zeros did not differ from the zeros expected in standardized residuals (all $p > 0.05$). Data are available in the Figshare repository https://figshare.com/s/9e0723e401d2356e7490.

3. Results

We calculated EPGs for both strongylids and tapeworms in 194 mountain gorilla fecal samples. The prevalence was 73% (median EPG = 16; range 0–1385) for strongylids and 77% (median EPG = 53; range 0–865) for tapeworms.

Strongylid EPG increased with increasing PC1 (estimate ± S.E. = 0.76 ± 0.11, Table 3, Fig. 3a), PC2 (estimate ± S.E. = 0.51 ± 0.16, Table 3, Fig. 3b) and relative density of gorillas (estimate ± S.E. = 0.35 ± 0.17, Table 3, Fig. 3c) and decreasing size of MCP (estimate ± S.E. = −0.34 ± 0.08, Table 3, Fig. 3d). The positive association between strongylid EPG and PC1 suggests that strongylid EPG increased with

Table 3
Outputs of the global (full) versus best-supported (minimal) models of effects that influence strongylid and tapeworm egg counts (per gram) in fecal samples obtained during Virunga 2015–2016 population survey of mountain gorillas. PC1 and PC2 represent the first two dimensions of principal components for vegetation and climate characteristics (see *Statistical analyses* for details). Status = monitoring (habituation) status, Density = mean relative density of gorillas per MCP, PC1 = first principal component, PC2 = second principal component, MCP = area of 500-m buffered minimum convex polygon of detected nest sites per gorilla group (see *Minimum convex polygon calculation* and *Statistical analyses* for details).

Effect	Full model		Minimal model	
Strongylids	*Df = 18, AIC = 1721.7*		*Df = 8, AIC = 1712.8*	
	Chi-square	*P value*	*Chi-square*	*P value*
Monitoring status	5.6	0.02	–	–
Density	3.9	0.05	4.0	**0.045**
PC1	2.8	0.10	52.5	**<0.001**
PC2	1.5	0.22	10.1	**0.001**
Group size	5.0	0.02	–	–
MCP	7.9	0.005	20.0	**<0.001**
Status*Density	1.8	0.18	–	–
Status*PC1	1.7	0.19	–	–
Status*PC2	1.0	0.32	–	–
Status*MCP	6.6	0.01	–	–
PC1*MCP	0.1	0.72	–	–
PC1*Density	1.2	0.27	–	–
PC2*MCP	1.2	0.28	–	–
PC2*Density	2.1	0.15	–	–
Tapeworms	*Df = 18, AIC = 1908*		*Df = 10, AIC = 1926*	
	Chi-square	*P value*	*Chi-square*	*P value*
Monitoring status	24.5	<0.001	0.94	0.33
Density	1.8	0.18	–	–
PC1	0.0	0.98	2.2	0.14
PC2	20.0	<0.001	6.1	**0.01**
Group size	3.2	0.07	–	–
MCP	22.5	<0.001	0.5	0.47
Status*Density	10.9	<0.001	–	–
Status*PC1	25.8	<0.001	6.6	**0.01**
Status*PC2	1.9	0.17	–	–
Status*MCP	28.5	<0.001	–	–
PC1*MCP	17.2	<0.001	5.0	**0.03**
PC1*Density	14.6	<0.001	–	–
PC2*MCP	13.1	<0.001	–	–
PC2*Density	1.4	0.24	–	–

decreasing temperature and decreasing % of open and closed mixed forests and *Neoboutonia* forest within MCPs. Conversely, the relationship of strongylid EPG to PC1 suggests that strongylid EPG increased with increasing mean elevation, annual precipitation, and % of *Hagenia* forest and *Hypericum* woodland within MCPs. The positive association between strongylid EPG and PC2 suggests that strongylid EPG increased with increasing % of bamboo and decreasing % of *Hypericum* woodland and *Neoboutonia* forest, open and closed mixed forests within MCPs.

Tapeworm EPG increased with PC2 (estimate ± S.E. = 0.35 ± 0.17, Table 3, Fig. 4a), which suggested that tapeworm EPG increased with increasing % of bamboo within MCPs and decreasing % of *Hypericum* woodland and *Neoboutonia* forest, open and closed mixed forests within MCPs. PC1 and MCP size had an interactive effect on tapeworm EPG values (Table 3, Fig. 4b). Specifically, tapeworm EPG increased with increasing PC1 if MCPs were smaller but decreased with increasing PC1 if MCPs were larger. This interaction suggests that in habitats with high % of *Hagenia* forest and *Hypericum* woodland (at lower temperature and with higher annual precipitation) tapeworm EPG increased with decreasing area of MCP, while in habitats with high % of open and closed mixed forest (with higher temperature) tapeworm EPG increased with increasing MCP area. PC1 and monitoring (habituation) status showed an interactive effect on tapeworm EPG; tapeworm EPG was positively associated with PC1 in unmonitored groups but slightly negatively associated in monitored groups. This interaction suggests that, in unmonitored groups, tapeworm EPG increased with decreasing mean annual temperature and decreasing % of mixed forest but increased with increasing mean elevation, mean annual precipitation and increasing % of *Hagenia* forest, *Hypericum* woodland, while in monitored groups the relationships are the opposite (Table 3, Fig. 4c).

4. Discussion

Present results support and substantially build upon the understanding generated from our previous study that was conducted on monitored mountain gorillas in both the Virunga Massif and Bwindi-Impenetrable National Park (Petrželková et al., 2021). As the previous study was focused on habituated (monitored) gorillas only, the potential impact of habituation on helminth infections remained unclear. Here we did not detect significant effect of monitoring (habituation) on strongylid infection; the impact of monitoring was detected only in tapeworm infections when interacting with environmental variables. Petrželková et al. (2021) only briefly discussed the impact of environmental factors (expressed as differences between sectors) on helminth infections. In this study, data obtained during the Virunga 2015–2016 population survey of mountain gorillas allowed us to focus on the impact of various environmental factors on strongylid and tapeworm intensities – either directly or via the impact on the host – thereby highlighting the importance of the epidemiological triad, the interactions between the helminth, host and environment. Our findings suggest that environmental characteristics (precipitation, temperature, elevation and vegetation type) are important drivers of intensities of strongylid and tapeworm infections in mountain gorillas in the Virunga Massif. Moreover, strongylid intensities were associated positively with mean relative density of gorillas and negatively with the size of their MCP.

The influence of vegetation cover types and climatic effects on strongylid egg counts suggests that strongylid egg counts increase with increasing precipitation and decrease with increasing temperature within MCPs. This finding was not surprising, as temperature and humidity are crucial survival parameters for free-living stages of strongylid nematodes (O'Connor et al., 2006). However, optimal climatic conditions for strongylid development can differ among strongylid genera (Pandey, 1972; Young et al., 1980; van Dijk and Morgan, 2008). Most studies exploring the effects of climatic conditions on strongylids have been conducted in livestock (e.g. Rossanigo and Gruner, 1996; Stromberg et al., 2012). In contrast, knowledge about the impact of temperature and precipitation on strongylids in free-ranging herbivores in the tropics is limited, with the exception of the research on the nematodes of wild African elephants (Condy, 1974), which demonstrated that larvae survive longer and more efficiently in the shade of trees and in soils of small particle size.

Research on the impact of climatic factors on strongylid infections in non-human primates is mostly restricted to studies exploring differences in helminth infections with seasonality (wet vs. dry season) and with contradictory conclusions (Huffman et al., 1997; Rothman et al., 2008; MacIntosh et al., 2010; Pafčo et al., 2017). Such conflicting results may be explained by differences in the composition of strongylid communities across non-human primates (Pafčo et al., 2018, 2019) and further complicated by the lack of knowledge about the impact of climatic factors on the strongylid species that occur only in wildlife including non-human primates. Our results show that strongylid communities are sensitive to varying precipitation and temperature across the study area. Higher precipitation at higher elevations may accelerate development of strongylids from an un-embryonated to an embryonated stage before hatching to the first of three larval stages (L1, L2 and L3) and especially L3 migration from the feces onto vegetation from where it can be ingested by a new host (Nielsen et al., 2007). Warmer temperatures also boost parasite development from egg to the infective L3 stage: under tropical conditions, development is rapid and a large number of L3 can be produced, although the L3 lifespan is short (Knapp-Lawitzke et al., 2016). We speculate that strongylid larvae may benefit from the lower

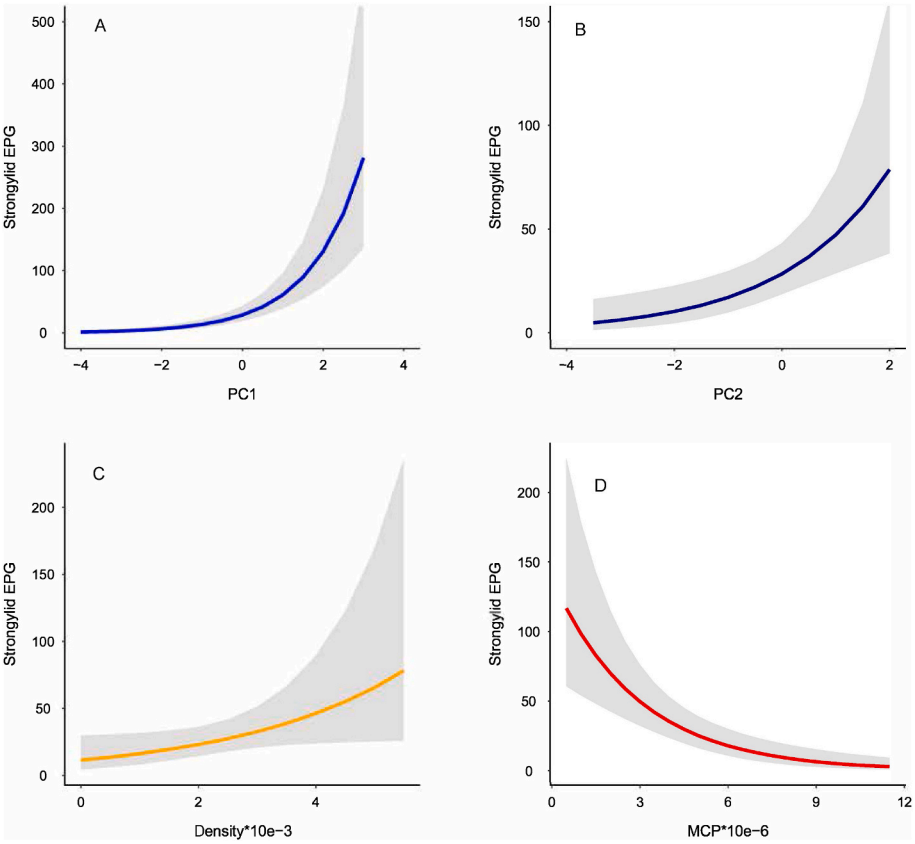


Fig. 3. Predicted lines from a generalized linear mixed model for significant effects of (a) the first (PC1) and (b) second principal component (PC2), (c) Density = mean relative density of gorillas per MCP and (d) MCP = area of 500-m buffered minimum convex polygon of detected nest sites per gorilla group, (see *Minimum convex polygon calculation* and *Statistical analyses* for details) on strongylid infection (egg counts per gram in fecal sample). Principal components were computed from 10 correlated environmental variables (see *Material and methods* for details).

temperatures at higher elevations in the Virunga Massif by going into an immotile state that allows conservation of energy (van Dijk et al., 2010), thereby prolonging their survival and leading to subsequently higher intensities of strongylid infections.

Significantly higher strongylid egg counts occurred in gorilla groups ranging in habitats with low proportions of open mixed, closed mixed, or *Neoboutonia* forests, and with concomitantly high proportions of other vegetation covers (*Hagenia* forest, *Hypericum* woodland, and pure and mixed bamboo). These results concur with our previous study (Petrželková et al., 2021), where the lowest strongylid egg counts corresponded to an area with a high proportion of open mixed forest, closed mixed forest, and *Neoboutonia* forests, namely, the Mikeno sector in the DRC.

In livestock, researchers found that the composition of pasture as well as length of grass could impact strongylid L3 occurrence (Callinan and Westcott, 1986; Marley et al., 2006), but to our knowledge no similar research has been done on the relationship between helminth infections and the vegetation in their habitats of any free-ranging herbivore hosts. Soil is an important refugium for L3s which also helps in preserving their fitness (Knapp-Lawitzke et al., 2016). L3 larvae need to be on vegetation to continue their life cycle, but it is assumed that the migration into the soil extends their lifespan as it protects L3 from dehydration, high temperatures and UV radiation (van Dijk et al., 2009; Chylinski et al., 2014). Increased growth of vegetation results in more compact cover, providing better shelter. Higher vegetation mass leads to reduced desiccation of soil (shelter from wind and sun) and also better protects the larvae affixed to vegetation against the above-mentioned factors (Knapp-Lawitzke et al., 2016). In the Virunga Massif, optimal vegetation growth (leading to highest biomass) occurs at an elevation range between 2000 and 3900 m (Kayiranga et al., 2017), not in higher elevations where higher strongylid egg counts occur. Despite this, we can still assume that *Hagenia* forest, *Hypericum* woodland and bamboo vegetation may provide better conditions for survival and migration of L3 strongylid larvae. Further research focusing on development from an un-embryonated egg to an embryonated stage, hatching to the first of three larval stages, as well as L3 larvae survival and migration in different gorilla habitat types is needed to understand how epidemiology of strongylid infections affects Virunga gorillas.

Importantly, although the vegetation types occurring in elevations >2000 m contain higher food biomass than in lower Virunga elevations (McNeilage, 2001), we cannot exclude the possibility that high elevation habitats with low temperatures and more precipitation may be suboptimal for gorillas leading to higher susceptibility to pathogens in gorillas ranging there as shown on strongylid nematodes in our study. Mountain gorillas are tolerant of a wide range of temperatures (Advani, 2014), but it is questionable to what extent today's elevation range and climatological niche represents their optimum (Thorne et al., 2013). Following extensive deforestation at lower elevations in Rwanda in the 1950s and 1960s, part of the Virunga gorilla population had to move to higher elevations, where temperatures can drop to 0 °C. Although Eckardt et al. (2019) revealed that neither exposure to cold temperatures nor to high elevations elevated baseline fecal glucocorticoid metabolite concentrations in mountain gorillas, they did not include gorilla groups ranging lower than 2300 m and thus further studies exploring the situation across the entire Virunga Massif are warranted.

We also cannot exclude that Virunga gorillas ranging in areas with a high proportion of mixed forest consume different diets that may support a higher nutritional plane, with more fruit consumed (as noted by McNeilage (1995), which may confer better immunity and lower infection rates (Hoste et al., 2005). Alternatively, those gorillas may specifically consume more medicinal plants. The consumption/availability of medicinal plants may vary across the Virunga vegetation zones. However, very little is known about self-medication or diet composition in the less studied portions of the Virunga Massif, especially on the Congolese side (McNeilage, 1995; Cousins and Huffman, 2002). Waterman et al. (1983) found that the vegetation in higher elevation

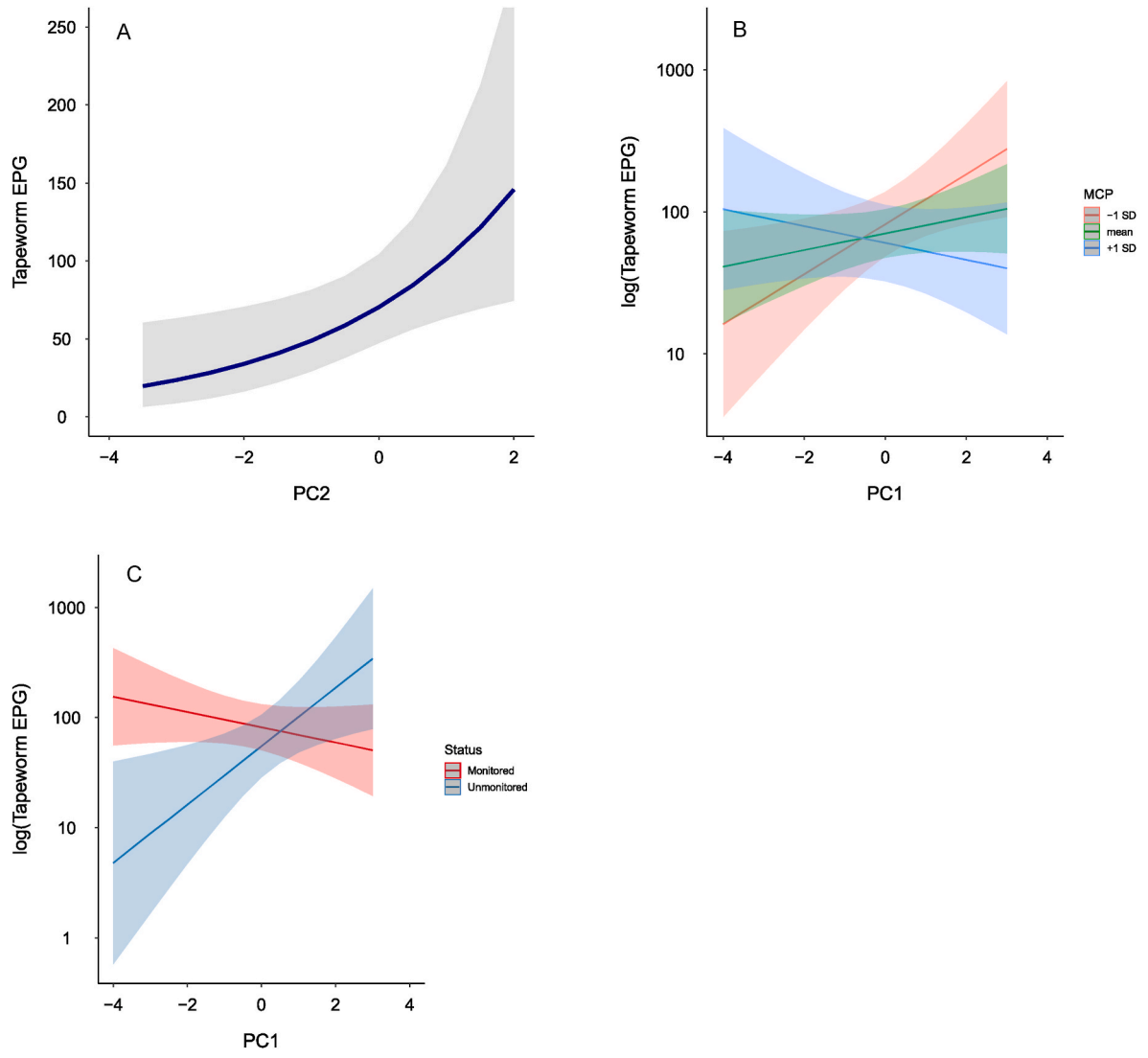


Fig. 4. Predicted lines from a generalized linear mixed model for significant effects of (a) the second principal component (PC2), (b) interaction between the first principal component (PC1) and MCP = area of 500-m buffered minimum convex polygon of detected nest sites per gorilla group, (see *Minimum convex polygon calculation* and *Statistical analyses* for details) and (c) interaction between monitoring (habituation) status and MCP on tapeworm infection (egg counts per gram in fecal sample). Principal components were computed from 10 correlated environmental variables (see *Material and methods* for details).

habitats around Mount Karisimbi and Mount Visoke contained low levels of plant secondary compounds (known for various biological effects) compared to other lower altitude forests, which may indicate a lower abundance of medicinal plants in those high elevations. Moreover, gorillas inhabiting mixed forest spend less time feeding on the ground and more time feeding arboreally (McNeilage, 1995), which may result in avoiding contact with infectious stages of strongylids and thereby reducing strongylid infection rates.

Our results provide further evidence that increasing gorilla densities and smaller MCPs are linked to higher intensities of strongylid infections as was proposed in our previous study (Petrželková et al., 2021). These results correspond to findings of many studies in both livestock and free-ranging herbivores showing that contact rates between hosts and strongylid transmission stages increase with host density (May and Anderson, 1978; Anderson and May 1979; Dobson, 1990; Arneberg et al., 1998). Although mountain gorillas groups have expanded and shifted their home ranges to forested areas with lower gorilla densities (Caillaud et al., 2020), being bounded by human settlements limits their ability to expand beyond the protected areas of the Virunga Massif, which has led to increasing population density, particularly in some areas. For example, in the Karisoke research area (area between Mount Karisimbi and Mount Visoke), the subpopulation increased in size by almost 50% and the number of groups tripled and groups had smaller annual home ranges compared to populations of other gorilla subspecies (Caillaud et al., 2014). In 2007, a series of group fission and formation led to a threefold increase in group density in the Karisoke area, whereas individual densities dropped due to groups expanding and shifting their home ranges to lower density areas [Fig. 3B in (Caillaud et al., 2020)]. Social behavior changes observed at high group densities with increased home range overlaps between groups affected the growth of this subpopulation. For example, a fivefold increase in the rate of infanticide and seven cases of lethal fights among mature males were recorded, and the annual subpopulation growth rate declined by half between 2000 and 2017 (Caillaud et al., 2020). Infanticide occurring during aggressive encounters between social units constituted 57% of the recent decline in the subpopulation growth rate. Other factors, such as feeding competition, male-male aggression, stress, and infectious disease, were also contributory factors in the subpopulation growth rate decline (Caillaud et al., 2020). Physiological studies revealed intergroup encounters to be a significant source of stress, increasing fecal glucocorticoid metabolite levels up to almost nine times, and this could influence susceptibility to pathogens, including strongylids (Eckardt et al., 2016, 2019). In the

Karisoke area, fatal gastritis cases accumulated after group density had markedly increased.

In our study, we were unable to investigate the relationship between group density and helminth infections due to insufficient spatial data of group locations to meaningfully represent the densities of groups in the landscape. Follow-up research on helminth infections should therefore consider effects of group density and associated changes in habitat use and social behavior, which may alter the exposure to infectious larvae in the environment and host susceptibility to disease. However, it is equally plausible that high strongylid intensities cause reduced movement and decrease the size of MCPs, because heavily infected gorillas may adjust their behavior as a result of their helminth burden and thereby moving less (Ghai et al., 2015). Further studies exploring the impact of the helminths on complexity in behavioral organization are warranted (Burgunder et al., 2017).

Helminths could regulate host populations if they reduce host survival and/or fecundity in density-dependent manner (May and Anderson, 1978; Anderson and May 1979; Tompkins et al., 2001). Increased occurrence of gastrointestinal diseases caused by helminths, some resulting in fatalities, calls for further research to elucidate if the Virunga mountain gorilla population could be regulated by strongylid nematodes as indicated by this and previous studies (Petrželková et al., 2021).

Understanding anoplocephalid tapeworm epidemiology is complicated due to their indirect life cycle involving an intermediate host, the soil oribatid mite (Denegri, 1993). Higher tapeworm infections were observed in gorillas using habitats with higher proportions of bamboo. The biological explanation of the interactions between PC1 (principal component reflecting proportion of *Hagenia* forest, mean elevation, mean annual temperature and precipitation) and MCP size or monitoring (habituation) status are complicated but point to an additional possible role of MCP size and habituation in shaping tapeworm infections.

However, environmental factors are likely principal drivers of tapeworm infections in mountain gorillas. Although intermediate oribatid hosts are known for 27 species of anoplocephalid tapeworms (Denegri, 1993), the exact mite species for anoplocephalid tapeworm transmission in mountain gorillas has not been identified. Various extrinsic factors, including elevation, temperature, humidity, soil characteristics and plant material entering the soil as litter (in the form of leaves, stems, and roots from vegetation cover), affect oribatid diversity and abundance (Marian et al., 2018; Villagomez et al., 2019; Ghosh, 2020; Sánchez-Galindo et al., 2021). Thus, we can assume that the intermediate oribatid host may be more abundant in particular elevations and vegetation types, which can lead to higher intensities of tapeworm infections in gorillas ranging especially in habitats with higher proportion of bamboo. Alternatively, higher intensities of tapeworm infections can be caused by the seasonal consumption of bamboo shoots, which might be more contaminated with soil (containing oribatid mites) than other food items. Further research focusing on the identification of intermediate oribatid hosts and their ecology to elucidate epidemiology of mountain gorilla tapeworm in the Virunga Massif is needed. Interestingly, in comparison to lowland gorillas, tapeworm infections in mountain gorillas are much more prevalent and intensities of infections are higher (Pafčo et al., 2017). That said, the impact of tapeworm infections on mountain gorilla health seems to be low (Muhangi et al., 2021; Petrželková et al., 2021), although this warrants further research.

5. Conclusions

New health challenges may be emerging as a result of the successful conservation of mountain gorillas, as evidenced by the steady increase in gorilla numbers in recent decades, but with limited possibilities for the gorillas to expand their range. We provide further support that gorilla densities together with pronounced differences in habitats across the Virunga Massif shape gorilla helminth infections. Our results suggest that the Virunga mountain gorilla population may be partially regulated by strongylid nematodes in some parts of the Virunga Massif. Mountain gorillas are at particular risk of high strongylid infection levels in selected areas of the Virunga Massif defined by higher precipitation, lower temperatures and particular vegetation types at higher elevations and if individual densities increase and size of their home ranges decrease. But impact of the ecological factors on helminth infections in the Virunga Massif needs to be verified using repeated fecal sampling as egg shedding is intermittent, in combination with long-term ranging data collected daily from monitored gorilla groups to more accurately calculate and represent home ranges and associated environmental variables. The results support previous findings in lowland gorillas that the effect of direct contact with humans (monitored vs. unmonitored groups) on helminth infections of great apes is negligible (Pafčo et al., 2017, 2019) compared to bacterial and viral infections (Ryan and Walsh, 2011), likely due to the complexity of parasite life cycles (including development outside of the hosts) and modes of transmission for infective stages. Further research activities should include i.e. proper taxonomic identification of whole strongylid communities, studies on ecology of strongylid larval stages in the various gorilla environments and identification of tapeworm intermediate hosts, oribatid mites.

Declaration of competing interest

The authors report no conflicts of interests.

Acknowledgement

The parasitological part of this study was supported by the Czech Science Foundation, Grant no. 18–24345S and the U.S. Fish and Wildlife Service Division of International Affairs, Grant no. F17AP00964. Klára Petrželková and Barbora Pafčo were also supported by institutional support of Institute of Vertebrate Biology, Czech Academy of Sciences (RVO:68081766). Computational resources were supplied by the project "e-Infrastruktura CZ" (e-INFRA LM2018140) provided within the program Projects of Large Research, Development and Innovations Infrastructures. The organization of Virunga 2015–2016 surveys was funded by the World Wildlife Fund for Nature, Fauna & Flora International (Laura J. Niles Foundation), Partners in Conservation/Columbus Zoo & Aquarium, Berggorilla & Regenwald Direkthilfe and The Max Planck Society, who did not contribute to the design, interpretation or the writing of this study. We thank Charles Kayijamahe, Gaspard Nzayisenga, Jacques Katutu and Eustrate Uzabaho for organizing supplies and samples and all partner organizations for their support and providing field staff for the sample collection during the Virunga 2015–2016 surveys, including the Greater Virunga Transboundary Collaboration, the Institute of Tropical Forest Conservation and the North Carolina Zoo. Data on group size and nest site locations were provided with permission from the Greater Virunga Transboundary Collaboration.

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Červená, B., Prokopová, T., Cameira, R.M., Pafčo, B., Samaš, P., Romportl, D., Uwamahoro, C., Noheri, J.B., Ntwari, A.E., Bahizi, M., Nzayisenga, G., Nziza, J., Gilardi, K., Eckardt, W., Ndagijimana, F., Mudakikwa, A., Muvunyi, R., Uwingeli, P., Cranfield, M., Šlapeta, J., Petrželková, K.J., Modrý, D.

2024

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Parasitology 151, 135–150. https://doi.org/10.1017/S0031182023001178

Parasitology

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Anoplocephalid tapeworms in mountain gorillas (*Gorilla beringei beringei*) inhabiting the Volcanoes National Park, Rwanda

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Research Article

[†]The authors mourn the death (August 28, 2023) of colleague Michael R. Cranfield, whose dedication to gorilla conservation and health was unparalleled.

Cite this article: Červená B *et al* (2024). Anoplocephalid tapeworms in mountain gorillas (*Gorilla beringei beringei*) inhabiting the Volcanoes National Park, Rwanda. *Parasitology* **151**, 135–150. https://doi.org/10.1017/S0031182023001178

Received: 17 May 2023
Revised: 15 November 2023
Accepted: 21 November 2023
First published online: 29 November 2023

Keywords:
Anoplocephala; Anoplocephalidae; *Bertiella*; *Gorilla beringei*; mountain gorilla; parasite ecology; parasite epidemiology; Rwanda

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Abstract

Cestodes of the family Anoplocephalidae parasitize a wide range of usually herbivorous hosts including e.g. rodents, ungulates, primates, elephants and hyraxes. While in some hosts, the epidemiology of the infection is well studied, information is lacking in others. In this study of mountain gorillas in the Virunga Massif, an extensive sample set comprising adult cestodes collected *via* necropsies, proglottids shed in feces, and finally, fecal samples from both night nests and identified individuals were analysed. *Anoplocephala gorillae* was the dominant cestode species detected in night nest samples and individually known gorillas, of which only 1 individual hosted a *Bertiella* sp. It was shown that the 2 species can be distinguished through microscopy based on egg morphology and polymerase chain reaction (PCR) assays for diagnostics of both species were provided. Sequences of mitochondrial (*cox 1*) and nuclear (ITS1, 18S rDNA, 28S rDNA) markers were used to evaluate the phylogenetic position of the 2 cestodes detected in mountain gorillas. Both types of fecal samples, from night nests and from identified individuals, provided comparable information about the prevalence of anoplocephalid cestodes, although the analysis of samples collected from identified gorilla individuals showed significant intra-individual fluctuation of *A. gorillae* egg shedding within a short period. Therefore, multiple samples should be examined to obtain reliable data for wildlife health management programmes, especially when application of anthelmintic treatment is considered. However, while *A. gorillae* is apparently a common symbiont of mountain gorillas, it does not seem to impair the health of its host.

Introduction

Cestodes of the family Anoplocephalidae which typically use mites as their intermediate hosts parasitize various vertebrates, typically herbivorous, across the globe (Beveridge, 1994; Deplazes *et al*., 2016). The genus *Anoplocephala* Blanchard, 1848 is known from a wide range of hosts including perissodactyls, primates, elephants and hyraxes, which possibly suggests a long association between hosts and parasites (Beveridge, 1994). On the other hand, there is considerable evidence for multiple host switching in the taxa parasitizing rodents and transfer among primates, dermopterans and marsupials has been recorded for the members of the genus *Bertiella* Stiles & Hassall, 1902 and *Progamotaenia* Nybelin, 1917 (Beveridge, 1985*a*, 1994; Haukisalmi *et al*., 2004, 2008, 2009; Wickström *et al*., 2005; Hardman *et al*., 2012). These cestodes are characterized mainly by an absent rostellum and unarmed suckers, a condition that is probably plesiomorphic and the 4 subfamilies could be a polyphyletic assemblage of taxa (Beveridge, 1994) as already confirmed by phylogeny of partial *cox1* (Sharma *et al*., 2016).

Besides a range of studies on anoplocephalids in Arctic rodents (Haukisalmi *et al*., 2001, 2004, 2008, 2009; Wickström *et al*., 2005) and Australian marsupials (Beveridge, 1985*a*, 1985*b*; Beveridge *et al*., 2007; Hardman *et al*., 2012), *Anoplocephala perfoliata* infections have been well studied in horses due to growing incidence of resulting disease (Meana *et al*., 2005; Back *et al*., 2013). Great apes, including humans, and other primates from

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South America, Africa and South-East Asia are most commonly hosts of *Bertiella*, *Anoplocephala* and *Moniezia* Blanchard, 1891, but occasional infections by *Mathevotaenia* Akhumian, 1946 and *Thysanotaenia* Beddard, 1911 have also been reported (Beveridge, 1994; Sapp and Bradbury, 2020). Occurrence of anoplocephalids in free-ranging non-human primate populations is usually not associated with clinical disease (Doležalová, 2018; Sapp and Bradbury, 2020), although gastrointestinal disturbances, diarrhoea, abdominal pain, anorexia, weight loss, vomit and constipation are reported from humans (Sapp and Bradbury, 2020).

In 1927, Nybelin described *Anoplocephala gorillae* based on material collected from a necropsied mountain gorilla (*Gorilla beringei beringei*) in the region of Mount Sabiniyo in Virunga range (Nybelin, 1927). Ever since, the parasite has been reported in all parasite surveys conducted in both the Bwindi and Virunga mountain gorilla populations, based mainly on presence of typical anoplocephalid eggs and/or detection of proglottids (Redmond, 1983; Ashford *et al*., 1990, 1996; Hastings *et al*., 1992; Sleeman *et al*., 2000; Mudakikwa *et al*., 2001; Kalema-Zikusoka *et al*., 2005; Rothman *et al*., 2008; Petrželková *et al*., 2021). Certain aspects of the ecology/epidemiology of cestode infection were studied in the Bwindi population by Ashford *et al*. (1996). However, only Doležalová *et al*. (2015) attempted to characterize mountain gorilla cestodes using DNA markers supporting identification at the species-level. The sequences of nuclear (ITS2 and 28S rRNA) and mitochondrial (*cox1* and *nad1*) genes obtained from anoplocephalid eggs from mountain gorilla feces clustered with primate *Bertiella* spp., and thus opened a question about spectrum of anoplocephalid cestodes occurring in mountain gorillas (Doležalová *et al*., 2015).

Our overall research has aimed to better understand the degree to which parasite burdens contribute to morbidity and mortality in this endangered great ape and patterns of helminth infections have been analysed across the mountain gorilla population (Petrželková *et al*., 2021, 2022). While strongylid egg counts significantly differed among individual sectors and vegetation types, no clear patterns were found for cestode infections (Petrželková *et al*., 2021, 2022). Therefore, the present study aimed to provide a deeper insight into selected aspects of the tapeworm infections in a sub-population of *G. beringei beringei* in the Volcanoes National Park (VoNP), the Rwandan part of the Virunga Massif. The specific aims of the study were: (i) improvement of diagnostic tools for detection of anoplocephalid cestodes in mountain gorillas, (ii) determining and characterizing the anoplocephalid cestodes in mountain gorillas based on adults collected from necropsies and proglottids and eggs shed in feces, including morphological features and genetic diversity, and, (iii) describing the epidemiology of the infections by analysing the prevalence and variation in egg shedding within and among the mountain gorilla individuals and groups in the VoNP, Rwanda.

Materials and methods

Study site

The study was conducted in the VoNP, the Rwandan section of a complex of protected areas spanning the borders of Rwanda, Uganda and the Democratic Republic of the Congo (DRC) called the Virunga Massif (451 km^2). The habitat in the VoNP has been characterized in detail elsewhere (Owiunji *et al*., 2005; Hickey *et al*., 2019; Petrželková *et al*., 2021), but briefly, the altitude, ranging between 1600 and 4500 m, determines various vegetation types (Fig. 1) and gorillas normally range in zones where temperatures drop to 0°C. The park's climate consists of 2 rainy (March–May and September–November) and 2 dry seasons (December–February and June–August). The VoNP area is surrounded by a dense human population of up to 1000 people km^{-2} (Bush *et al*., 2010), whereas the mountain gorilla density in the VoNP is estimated to be 1.7 individuals per km^2 (Hickey *et al*., 2019).

Fecal sample collection

Nineteen habituated mountain gorilla groups ranging in the VoNP with an estimated 278 individually known gorillas were targeted in 2018–2019. The location, demography, and behaviour of these groups are monitored on a daily basis by the Dian Fossey Gorilla Fund (DFGF) and Rwanda Development Board (RDB) park rangers, while the health of the gorillas is regularly monitored by Gorilla Doctors (GD) veterinarians and daily by park personnel (DFGF and RDB) trained by the GD. GD also provide *in situ* veterinary care to mountain gorillas. Data on group size (number of individuals), age and sex of individually sampled gorillas were obtained from RDB and DFGF.

In 2018 and 2019, fresh fecal samples were collected from habituated mountain gorilla groups by trained staff of RDB, DFGF and GD across the VoNP in 4 sampling periods: in the dry seasons (December–February and June–August), and in the wet seasons (March–May and September–November). Two types of fecal samples were collected: night nest samples from the previous night and samples from identified individuals obtained during group visits. The methodology of nest and individual sample collection is described in detail elsewhere (Petrželková *et al*., 2021; Sinayitutse *et al*., 2021). All samples were placed into plastic bags, labelled with date and time of collection and group name. The samples were also labelled with name of the individual (when available) and sex/age class (dominant silverback/silverback >12 years; blackback 8–12 years; adult >8 years; subadult/juvenile 3.5–8 years; infant 0–3.5 years) (Sinayitutse *et al*., 2021).

Fecal samples (n = 1500) were immediately transported to the GD field laboratory in Musanze, stored in a refrigerator, and examined within a maximum of 48 hours after defecation. Any unusual appearance of the feces, such as diarrhoea or presence of tapeworm proglottids (Fig. 2A) was recorded. Proglottids found in feces were collected and stored in 96% pure ethanol. Three sub-samples of feces were taken: (i) 1 g of feces was preserved in 96% ethanol for DNA analyses (ii) a total of 3 g of feces were preserved in formalin for storage and potential further microscopic examination; and (iii) the last aliquot was immediately processed by Mini FLOTAC© (see below).

Coproscopic analyses and parasite quantification

Anoplocephalid tapeworm eggs in fresh feces were quantified *in situ* by Mini-FLOTAC© as described in Petrželková *et al*. (2021). The egg counts expressed as eggs per gram of feces (EPG) were recorded. When a sample was evaluated as cestode-negative by Mini-FLOTAC©, the formalin-preserved aliquot was re-examined by a sedimentation-based technique (Doležalová *et al*., 2018) providing the quantification as eggs per gram of fecal sediment (EPGS). The EPGS is not equal to the EPG as the fecal sediment obtained after sieving of the feces does not contain the bigger, usually fibrous particles. In our experience, EPGS is about 1.7 times higher than EPG (data not shown). We took the weight of fecal sediment during the Mini-FLOTAC© examination, which enabled us to calculate EPGS also for the samples examined *in situ* by Mini-FLOTAC©.

Egg morphology analyses

Formalin-preserved aliquots of samples identified as *A. gorillae* or *Bertiella* sp. positive based on DNA barcoding (see below) were

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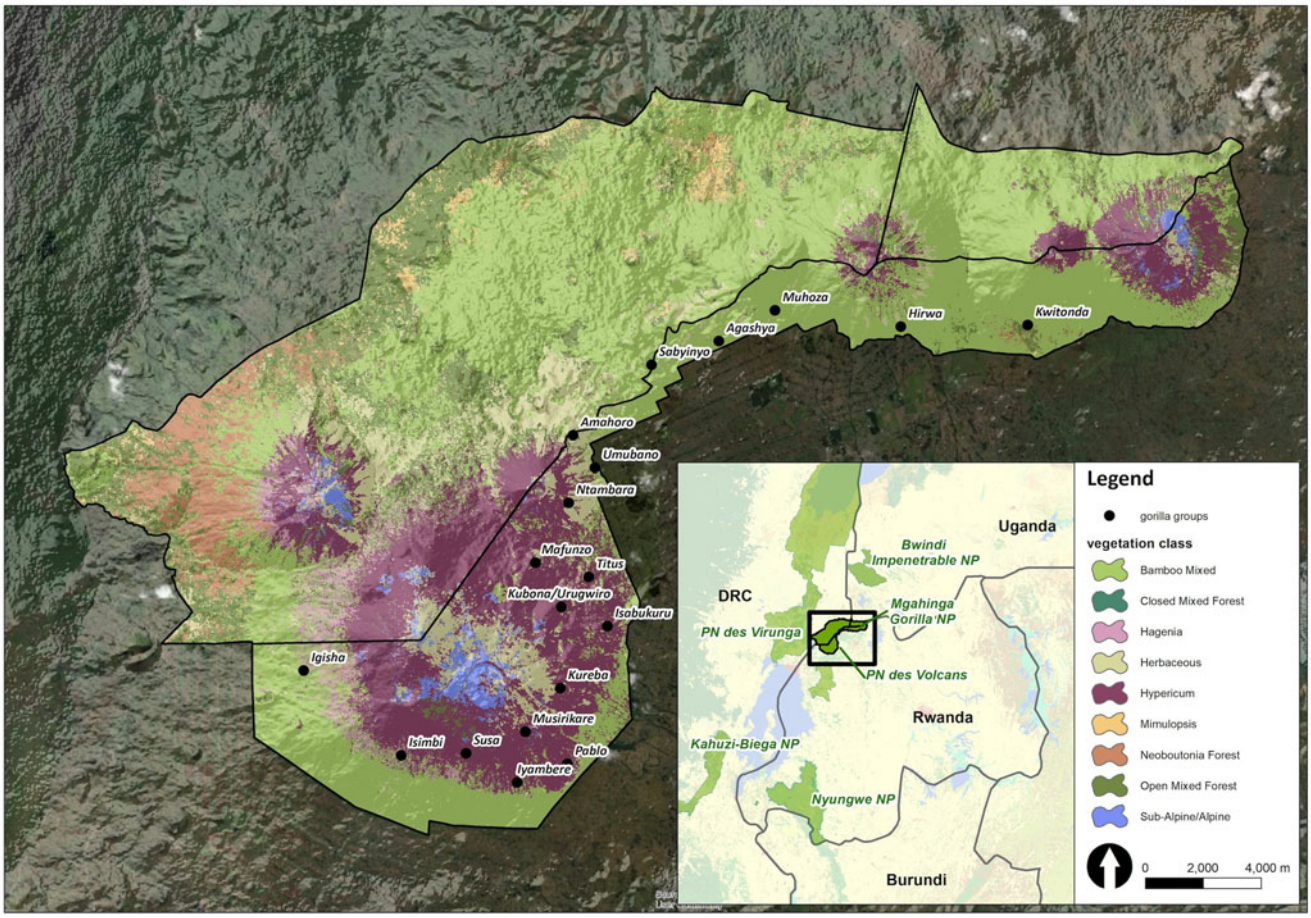


Figure 1. Map of Virunga Massif region picturing the studied gorilla group distribution and vegetation types. Adapted from Petrželková *et al.* (2021).

used for detailed egg morphology investigation. Modified Sheather's sugar flotation technique (Jirků-Pomajbíková and Hůzová, 2018) was used to concentrate the eggs. Egg measurements and microphotographs were taken using an Olympus BX53 microscope equipped with Nomarski differential contrast, an Olympus DP73 digital camera and CellSense Dimension software (Olympus, Tokyo, Japan). Maximum egg and oncosphere dimensions (Fig. 3) and type of pyriform apparatus (PA) classified using a scale from 1 to 10, where 1 refers to *Bertiella*-like PA (brush like, forming thin filaments), whereas 10 refers to the *Anoplocephala*-like PA (bifurcated), were recorded. To evaluate the morphological differences between *Anoplocephala* and *Bertiella* eggs statistically, we used a general linear mixed model with normal distribution (hereafter: LMM).

Adult cestodes collection

As a part of veterinary surveillance, all deceased animals that are recovered from the forest are necropsied by GD veterinarians following standardized protocols. Various tissue and other biological samples (e.g., ingesta, feces, urine) are collected, including adult parasites observed anywhere in the carcass (Fig. 2B). For purposes of this study, a collection of 53 adult cestodes (obtained from 5 gorillas between 2015 and 2018) from the GD biobank was available. Adult anoplocephalid cestodes were collected mainly from the small intestine, but also from the caecum and colon. The specimens were subsequently preserved in 96% ethanol or 10% formalin. The morphology of adults was studied using a stereomicroscope Olympus SZ51 and a microscope Olympus

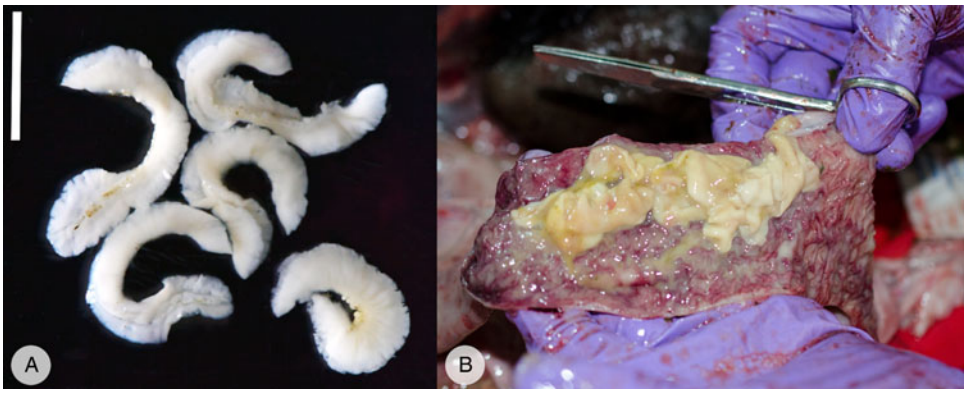


Figure 2. Anoplocephalid cestodes collected from mountain gorillas. (A) Individual proglottids recovered from mountain gorilla feces. Scale 1 cm. (B) Adults inside the mountain gorilla small intestine as recovered at necropsy.

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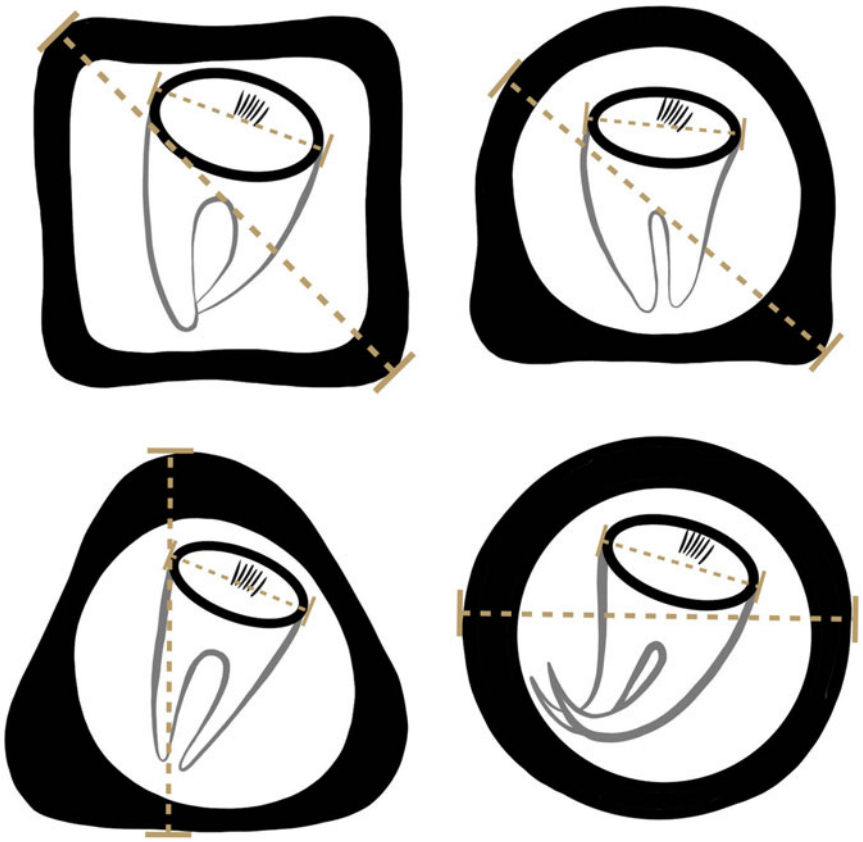


Figure 3. Depicting of how the measurements were taken from anoplocephalid eggs. As many anoplocephalid eggs are asymmetrical, egg and oncospherical diagonals (dashed lines) were introduced as maximal dimensions used for morphology investigation.

BX53 (Olympus, Tokyo, Japan). The specimens were determined using taxonomic keys and available publications (Nybelin, 1927; Stunkard, 1962; Beveridge, 1994; Sapp and Bradbury, 2020; Servián *et al.*, 2020).

DNA extraction

DNA was extracted from feces, from proglottids obtained from gorilla feces and from adult cestodes, all stored in ethanol. For fecal samples, aliquots of 0.5 g of feces were put in a thermoblock at 37°C overnight for ethanol evaporation. Then, the DNA was extracted using the DNeasy PowerSoil Pro Kit (Qiagen, Hilden, Germany), with a single modification to the manufacturer's instructions: the incubation steps were prolonged to 30 min. For the proglottids and adult cestodes, approximately 1 cm of proglottid tissue was minced and the total genomic DNA was extracted by NucleoSpin Tissue Kit (Macherey–Nagel, Düren, Germany). The tissue was lysed in Proteinase K overnight and the DNA was eluted to 100 μL of elution buffer. An adult of *A. perfoliata* from a horse from the Czech Republic was used as comparative material. This tapeworm was obtained during a standard necropsy performed at the Department of Pathology and Parasitology, Faculty of Veterinary Medicine, University of Veterinary Sciences Brno and preserved in 96% ethanol until further processing.

Nuclear ribosomal and mitochondrial DNA amplification

DNA extracted from adult cestodes was used for amplification of partial small ribosomal subunit gene (18S rDNA) and the region spanning the internal transcribed spacer 1 (ITS1), 5.8S rRNA (5.8S) gene, and internal transcribed spacer 2 (ITS2). Partial 18S rDNA and complete ITS1 were amplified in all collected proglottids. A subset of proglottids obtained from feces and adult tapeworms was used for amplification of partial cytochrome c oxidase subunit 1 gene (*cox1*) and gene for large ribosomal subunit (28S rDNA). Detailed information on primers, polymerase chain reaction (PCR) reaction composition and cycling conditions is provided in supplementary files, Table ST1. All PCR products were separated in 1% agarose gel with Midori Green (NIPPON Genetics EUROPE, Germany) and visualized on an UV illuminator. The PCR products were purified using Gel/PCR DNA Fragments Kit (Geneaid, New Taipei City, Taiwan) either directly or from bands cut from the gel and sent for commercial Sanger sequencing (Macrogen Europe B.V., Amsterdam, Netherlands).

PCR assays for detection of anoplocephalid tapeworms

The nested PCR protocol described by Drogemuller *et al.* (2004) for *A. perfoliata* was tested using the DNA extracted from 2 adult gorilla tapeworms and 2 proglottids from feces as the source of template DNA. The external primers S18 (F) and L3 T (R) amplify the whole ITS1, 5.8S and ITS2 region, while the internal primers AP-ITS2-2F and AP-ITS2-3R target ITS2 only. The PCR reaction composition and cycling conditions are specified in supplementary material (Table ST1). When a band of corresponding size and Sanger sequencing confirmed a successful DNA amplification from adult cestodes, a set of 10 samples of DNA extracted from fecal samples with various anoplocephalid cestode EPG scores (>1000 EPG, $n = 4$; <100 EPG, $n = 6$) was used to test applicability of the assay on fecal DNA. All amplicons were sequenced to confirm the specificity of the assay.

While testing this PCR assay, 1 sample did not produce any band, despite having a high EPG value. Using less specific primers targeting the ITS1 region (McLennan *et al.*, 2017) (Table ST1), we obtained a 300 bp amplicon sequence that revealed presence of a *Bertiella* sp. in this sample (see details in the Results section, Chapter '*Bertiella* sp. detected in one mountain gorilla individual'). Using newly obtained *Bertiella* and *Anoplocephala* sequences and sequences of *Anoplocephala* and *Bertiella* spp. available in

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GenBank, *Bertiella*-specific primers were designed in Geneious Prime v.2021.0.1 (https://www.geneious.com). The PCR assay was optimized, and its specificity tested by using the DNA from *Anoplocephala* proglottids and from *Anoplocephala*-positive and *Bertiella*-positive fecal samples (Table ST2).

A subset of cestode-negative samples as revealed by Mini-FLOTAC© examination was examined by both PCR assays and sedimentation method (samples listed in supplementary material Table ST2) to compare the sensitivity of both diagnostic approaches.

Sequences and phylogenetic analyses

All sequences obtained were checked for quality and trimmed manually using Geneious Prime v.2021.0.1 (https://www.geneious.com). The identity of sequences was validated using the BLAST software (Basic Local Alignment Search Tool; Altschul *et al*., 1990). The alignments were performed by Clustal Omega implemented in Geneious Prime and the same software was used for calculation of pairwise sequence distances. For the phylogenetic analyses, sequences of available anoplocephalid tapeworms (18S rDNA, 28S rDNA, ITS1, *cox1*) were downloaded from GenBank and aligned with our sequences (Supplementary file SF5). The maximum-likelihood phylogenetic trees were calculated by IQ-TREE (Trifinopoulos *et al*., 2016). The most suitable model was chosen by ModelFinder (Kalyaanamoorthy *et al*., 2017) implemented in IQ-TREE based on the highest Bayesian information criterion scores and weights. The tree topology was tested by 1000 replicates of ultrafast bootstrap (Minh *et al*., 2013) and Shimodaira–Hasegawa (SH)-like approximate likelihood ratio test (Anisimova *et al*., 2011). The trees were visualized using the software iTOL v6 (Letunic and Bork, 2021).

Results

Proglottids and adult cestodes confirmed to be A. gorillae

Fifty-three adult tapeworms originating from 5 individual gorillas were examined. The length of strobilae varied between 1.5 and 13 cm by 0.8–1.4 cm in width. Four circular suckers (2 mm in diameter) were observed terminally on a small scolex (Fig. 4A). The proglottids in the strobilae were extended transversely, significantly wider than longer (length 0.5–2 mm, width 8–14 mm) and craspedote. No signs of a rostellum were observed (Fig. 4B). The individual proglottids collected from feces were usually curled or folded (Fig. 2A), on average 4 mm long and 15 mm wide, thin and had undulated edges. A collection of specimens used in this study was deposited to the Museum of Environment, Karongi District, Rwanda.

In total, 1500 fecal samples were collected in 2018 and 2019 (Fig. 5), and 69 proglottids from 69 fecal samples were retrieved. More proglottids in 1 fecal sample were observed sometimes, however the number of proglottids was not recorded and only 1 proglottid per sample was preserved. Five proglottids were compressed between 2 microscope slides to examine the eggs, but no eggs were observed. Single genital pore was distinguished in all 5 proglottids. All listed morphological features corresponded to the morphology of *A. gorillae* as described by Nybelin (1927). Egg counts in the fecal samples containing proglottids varied from 15 to 8190 EPG (eggs per gram of feces) and most feces looked normal (i.e., not diarrhoeic or haemorrhagic), only in 1 case, presence of mucus was recorded.

PCR detected more cestode-positive samples than microscopy

The nested-PCR assay originally designed to target the ITS2 region of *A. perfoliata* was efficient in *A. gorillae* DNA amplification, despite 1 mismatch in each of the internal primers. Amplicons of expected size were obtained using DNA extracted from samples with 8 to 4260 EPG and no non-specific amplification was detected. When using PCR assay targeting *Bertiella* ITS1 region, amplicons of expected size were obtained from samples containing *Bertiella* sp. eggs, while no amplicon was observed using DNA extracted from *Anoplocephala* spp. proglottids or from fecal samples containing *A. gorillae* DNA. Protocols for both PCR assays are specified in supplementary material Table S1.

Out of the 99 cestode-negative samples as detected by Mini-FLOTAC©, the sedimentation method revealed 14 samples containing cestode eggs in quantities ranging from 5 to 2139 eggs per gram of fecal sediment (EPGS) (Table S2, Fig. 5). In 12 of the 14 samples, egg morphology corresponded to *A. gorillae*, while eggs in the remaining 2 samples were of the *Bertiella* morphotype (see chapter on *Anoplocephala* and *Bertiella* egg morphology).

PCR assay targeting *Anoplocephala* detected 16 positive samples, of which 5 samples were initially evaluated as cestode negative by sedimentation (i.e., negative by 2 microscopy-based methods), while in the other 11 PCR-positive samples, *Anoplocephala* eggs were observed using the same technique (Fig. 5).

All amplicons obtained from fecal DNA samples were sequenced and were identical with ITS2 sequence obtained from adult *A. gorillae*. In 1 sample evaluated as *Anoplocephala*-positive by sedimentation (5 EPGS), no amplicon was obtained despite an increased effort (DNA was re-extracted, PCR was repeated with both increased and decreased DNA template volume).

Using the PCR assay targeting *Bertiella* ITS1, only 2 samples were detected as *Bertiella*-positive and in both, *Bertiella*-morphotype eggs were observed also in the sedimentation (Fig. 5). Sequences obtained from these samples were identical with the *Bertiella* sp. sequences from the original fecal sample (see the next chapter). In all remaining 97 samples, *Bertiella* DNA was not detected.

To conclude, in total 19 falsely cestode negative samples were detected, 14 *via* sedimentation method, while PCR (both assays

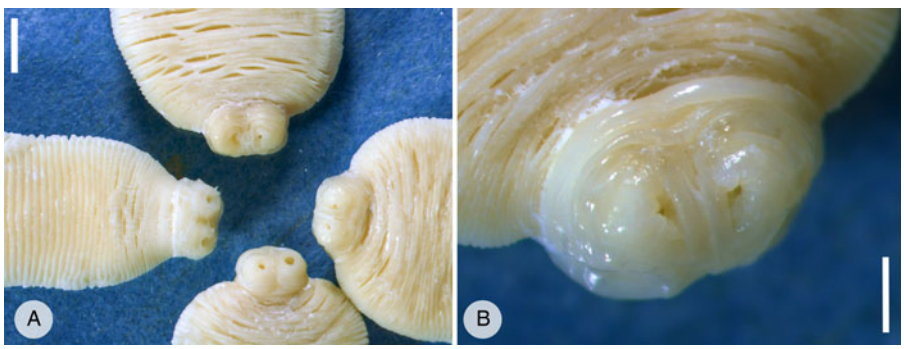


Figure 4. Adult *Anoplocephala* recovered from the mountain gorilla small intestines and preserved in ethanol. (A) Scolexes with 4 circular suckers, very short neck and proximal part of strobila. Note the craspedote proglottids, which are significantly wider than longer. Scale 0.5 cm. (B) Detail of scolex with circular suckers. No rudiments of a rostellum are visible. Scale 2 mm.

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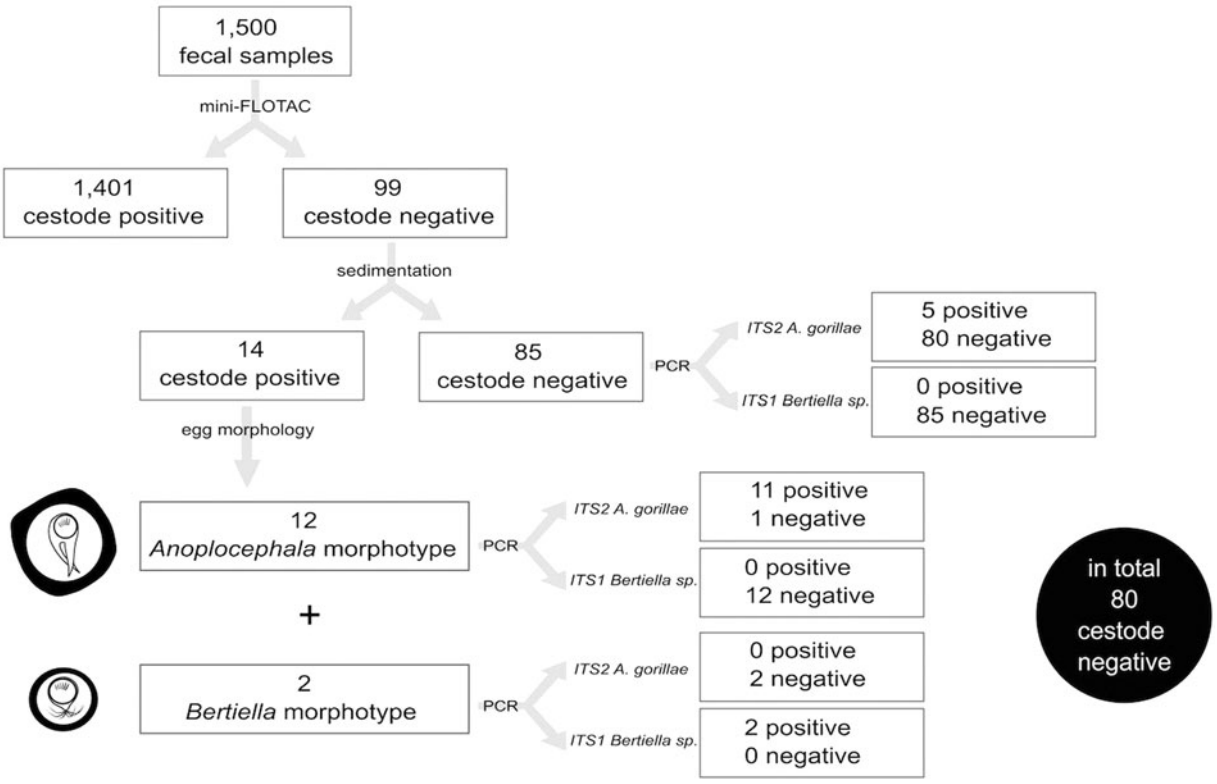


Figure 5. Diagram picturing the workflow of the anoplocephalid cestode diagnostic procedure in mountain gorilla fecal samples. Out of 99 Mini-FLOTAC©-cestode-negative samples, 19 were found as falsely-negative.

combined) identified 18 false negatives, including 5 samples evaluated as negative in sedimentation. The information about the 19 false negatives was used to complement the anoplocephalid cestode prevalence calculations.

Bertiella *sp. detected in 1 mountain gorilla individual*

While initially optimizing PCR assays for the detection of *A. gorillae*, no amplicon was obtained by *Anoplocephala*-specific primers in 1 sample, despite the high egg counts recorded. Primers targeting the ITS1 region of anoplocephalid tapeworms (McLennan *et al.*, 2017) amplified a sequence (683 bp) which differed by 41.1% from ITS1 sequences obtained from our *A. gorillae* proglottids and closely matched *Bertiella* sp. H_1 (Accession number MK993273.1, host: human, country: Argentina, 90.96% identity) in BLAST. Additionally, partial 18S rDNA (714 bp) was also amplified and sequenced from the sample to allow a better characterization of this taxon. In this nuclear marker, the pairwise sequence distance between the newly detected *Bertiella* sp. and *A. gorillae* was 18.5%. The formalin-preserved aliquot of this sample was re-examined by flotation, showing eggs with morphological features corresponding to *Bertiella* sp. as described below.

The *Bertiella*-positive sample originated from a mountain gorilla male infant named Inkingi from the Hirwa group. Firstly, all samples collected from this individual ($n = 4$; collected in 2 sampling periods 3 months apart) were tested to verify that the *Bertiella* eggs in feces were not just a spurious parasite. *Anoplocephala*-specific PCR assay was also applied to detect possible co-infection by both cestode species. Two Inkingi's samples were positive for *Bertiella* only, but both *A. gorillae* and *Bertiella* sp. DNA was detected in the remaining 2 samples. Subsequently, fecal samples from the other 15 members of Inkingi's family group, were tested for presence of *A. gorillae* and *Bertiella* sp. by PCR, using 2 samples with the highest EPG from each gorilla. All individuals tested positive for *A. gorillae* only. Therefore, *Bertiella* and *A. gorillae* prevalence among the Hirwa group individuals was 6.6 and 100% respectively. Inkingi was the only individual infected by both anoplocephalids. Finally, another 100 samples collected from different individuals from 16 study groups were randomly selected and tested with the *Bertiella*-specific PCR assay; all with negative results.

ITS1 and 18s rDNA phylogenies confirm 2 anoplocephalid cestodes parasitizing mountain gorillas

Representative sequences of complete ITS1, 5.8S and ITS2 (1876 bp) and partial 18S rDNA (654 bp) were obtained from 2 selected *A. gorillae* adults originating from 2 deceased gorillas from 2 different groups. No difference in either marker was observed. 18S rDNA and ITS1 sequences were also obtained from 51 proglottids. At least one of the markers was sequenced in 14 proglottids (ITS1 and 18S rDNA each in 7 samples), while amplification was not successful in 4 samples even though the DNA was re-extracted, DNA template volume in the PCR was increased and annealing temperature adjusted.

All sequences of 18S rDNA (583–715 bp) were identical to each other except for 1 sequence that differed in a single nucleotide, which was ambiguous (S in position 291 in the alignment). The majority of ITS1 sequences (326–642 bp; $n = 46$) was also identical to the sequence obtained from adults and this haplotype was labelled as Hap1. Five more variants of ITS1 were identified in our dataset (Hap2 – Hap6), each represented by a single sequence. The haplotypes differed in 1 to 6 nucleotides from each other. Eight sequences were too short to be assigned to either haplotype Hap1 or Hap2. ITS1 sequences obtained from fecal samples ($n = 5$) during the optimization of PCR assays were also identical with the haplotype Hap1. The sequences were

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uploaded to GenBank under accession numbers OR863079-91 and OR863669-74.

Final alignment of 18S rDNA (897 bp) comprised 6 sequences obtained from *Bertiella* sp. and *A. gorillae* specimens in this study and 22 anoplocephalid cestode sequences available in GenBank. The alignment of ITS1 (1137 bp) consisted of 9 sequences from our *Bertiella* sp. and *A. gorillae* samples and 90 more sequences of anoplocephalid cestodes downloaded from GenBank. The maximum likelihood phylogenetic trees were calculated by TPM2 + F + G4 and TPM2 + F + I + G4 for 18S rDNA and ITS1 respectively (Fig. 6A; Supplementary files SF1, SF2).

In both phylogenetic analyses, distinct clades of *Anoplocephala* spp. and *Bertiella* spp. were formed (Fig. 6A, Supplementary files SF1, SF2). All sequences originating from proglottids, and adult worms clustered with *Anoplocephala* spp. from equids. The difference between *A. gorillae* and *A. perfoliata* was 7.6–13.2 and 2.6–3.5% for ITS1 and 18S rDNA respectively. In the ITS1, *A. gorillae* differed from *A. magna* by 6.3–8.2%. Sequence obtained from the fecal sample with *Bertiella* eggs clustered within a clade comprising other *Bertiella* spp. sequences from non-human primates and humans (Fig. 6A), specifically in a sub-clade with sequences originating from a human and a howler monkey from Argentina, but the difference was about 11.4–11.7%. The sequences from Ugandan chimpanzees (Accession numbers LC185227-9; geographic proximity to our material) differed from our *Bertiella* sp. by 31.2–37.4%. In 18S rDNA phylogenetic tree (Supplementary file SF1), the mountain gorilla *Bertiella* clustered most closely with a sequence from a sanctuary chimpanzee from Kenya (MW741843; Supplementary file SF1), but the lowest pairwise distance (0.4%) was observed between our *Bertiella* and sequence of *B. studeri* from a monkey (most probably a long-tailed macaque) from Mauritius (GU323706). Previously published sequence of *Bertiella* sp. obtained from a mountain gorilla from Rwanda (MW741845) differed from our isolate by 2.1% and clustered in other *Bertiella* sub-clade (Supplementary file SF1).

Cox1 and 28s rDNA phylogenies confirm position of A. gorillae *in the genus Anoplocephala*

We obtained 11 sequences of partial *cox1* (459–1064 bp) from 9 adult cestodes and 2 proglottids isolated from feces. Four haplotypes differing by 1 to 4 nucleotides were detected. The BLAST search showed 89% similarity to *A. perfoliata* (accession number KR054960) for isolate T7, while the other isolates showed 89.74–89.85% similarity to *A. magna* (accession number KU236385). The sequences were uploaded to GenBank under accession numbers OR864671-76. The maximum likelihood phylogenetic tree (Fig. 6B, Supplementary file SF3) was calculated from an alignment (1127 bp) of 152 anoplocephalid cestode sequences, including 6 sequences representing our isolates from mountain gorilla by TIM2 + F + R5 model with 2 *Hymenolepis* spp. sequences used as outgroup.

In total, 5 sequences (1169–2011 bp) of 28S rDNA 5′-end were obtained from 3 adult tapeworms and 2 proglottids collected from feces. All sequences were identical. Only in 1 adult (isolate T2), 2 haplotypes differing in a single nucleotide deletion (G at position 704) were detected. BLAST search showed 97.98–98.05% similarity (71% coverage) to *A. perfoliata* isolate N54 (accession number AY569769). The sequences were uploaded to GenBank under accession numbers OR863675-80. The maximum likelihood phylogenetic tree (Fig. 7, Supplementary file SF4) was calculated from a 1910 bp alignment of 127 anoplocephalid cestode sequences and 2 *Hymenolepis* spp. sequences used as outgroups by the TPM3u + F + R3 model.

In both trees (Fig. 6B, Fig. 7, Supplementary files SF3, SF4), clades corresponding to distinct species were formed. Sequences from mountain gorillas' *A. gorillae* clustered in a separate clade close to other *Anoplocephala* spp. Surprisingly, *cox1* sequence of *A. manubriata* (accession number KU903287) from an Asian elephant did not cluster with other *Anoplocephala* spp. but with *Bertiella* spp. sequences obtained from primates, including humans (Fig. 6B, Supplementary file SF3). In 28S rDNA, the genus *Bertiella* appeared as a polyphyletic group (Fig. 7, Supplementary file SF4) with a clade comprising sequences from primates, including humans and a separate clade comprising sequences from marsupials.

Anoplocephala gorillae *and* Bertiella *sp. egg morphology differences summarized*

Three different situations were recorded within our sample set after microscopy and PCR: either (i) *A. gorillae* or (ii) *Bertiella* sp. were found as a mono-infection or (iii) co-infection of both species was detected in a sample. Five samples covering each situation were used for detailed egg morphology examination to investigate if the eggs of the 2 cestodes can be distinguished. In total 261 eggs from 5 gorilla individuals were examined, including 131 *Bertiella* eggs originating from a single gorilla individual and 130 *A. gorillae* eggs extracted from 4 individuals. Based on egg shape, maximum length, oncosphere length and PA, 2 egg morphotypes corresponding to *A. gorillae* and *Bertiella* sp. were distinguished (Fig. 8). *A. gorillae* egg length (mean = 67.0 μm, 95% CI 65.1–69.0, $n = 130$) was larger than in *Bertiella* sp. (mean = 48.2 μm, 95% CI 46.1–50.5; LMM: estimate ± S.E. = −0.020 ± 0.001, $t = -21.4$, $P = 0.001$). Oncosphere length did not differ between *A. gorillae* (mean = 16.0 μm, 95% CI 13.8–19.1) and *Bertiella* sp. (mean = 18.4 μm, 95% CI 14.6–24.8, $n = 131$; LMM: $t = -1.27$, $P = 0.27$). Eggshell thickness had a non-significant tendency to be larger in *A. gorillae* (mean = 6.5 μm, 95% CI 4.8–8.5, $n = 100$) than in *Bertiella* sp. (mean = 3.9 μm, 95% CI 2.0–7.2, $n = 100$; LMM: $t = -0.63$, $P = 0.08$). Similarly, the PA, classified on a scale from 1 (brush-like) to 10 (bifurcated), had a non-significant tendency to be more bifurcated in *A. gorillae* (mean = 8.0, 95% CI 6.7–9.2, $n = 98$) and rather brush-like in *Bertiella* sp. (mean = 3.6, 95% CI − 5.3–12.4, $n = 99$; LMM: $t = -8.70$, $P = 0.06$). Figure 8 summarizes the basic morphological characteristics and morphometry of *Anoplocephala* and *Bertiella* eggs.

Eggs of *A. gorillae* had various shapes, mostly quadrangular with a very thick eggshell, but a triangular or polygonal shape was also recorded. The oncosphere was mostly kidney-shaped surrounded by a clearly bifurcated PA. In *Bertiella* sp., oncospheres were mostly spherical and the egg wall was thinner than observed in eggs of *A. gorillae*. The PA of *Bertiella* sp. was branched into many thin fibres, resembling a brush (Fig. 9).

Anoplocephala gorillae *eggs detected in all studied groups with very variable EPG*

Anoplocephala gorillae eggs were detected in the vast majority (95%) of the 1500 fecal samples examined and the egg counts varied from 8 to 8190 eggs per gram of feces (EPG) with a median of 203 EPG. In total, 5.5% ($N = 82$) of samples exceeded 1000 EPG, and 1.3% ($N = 20$) samples had EPG higher than 2000. We observed a great variability of EPG values within and among groups (Fig. 10). The differences between groups were statistically significant – details in Petrželková *et al.*, 2021.

In total, 617 nest samples from 19 groups were collected and examined in 7 sampling events corresponding to dry and wet seasons of 2018 and 2019. However, the majority of groups was sampled only during the dry (Jan–Feb) and wet (Sep–Nov) season in 2018 (382 fecal samples; Table 1). As these samples do not originate from identified individuals, we avoid interpreting the

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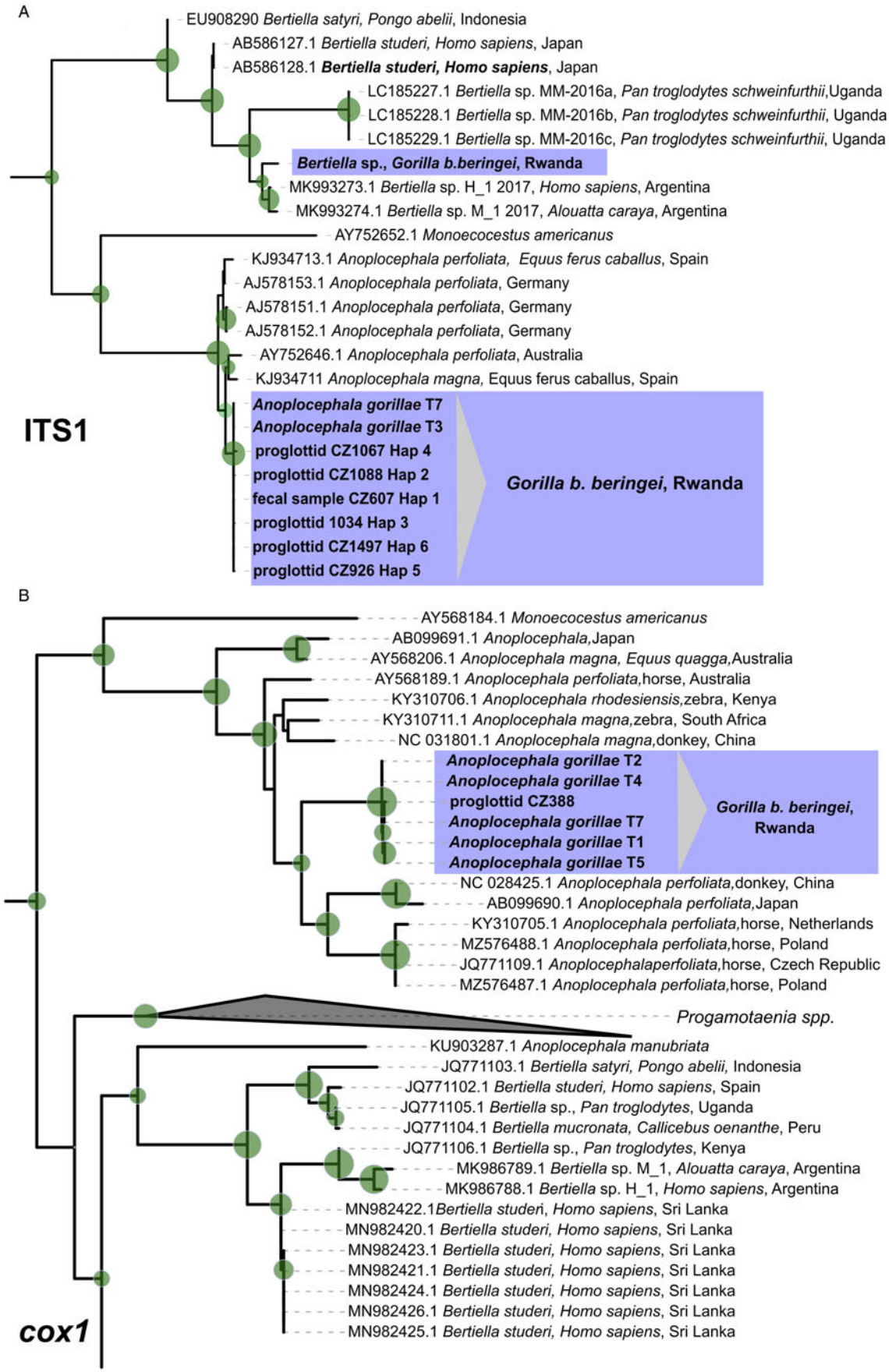


Figure 6. Cut outs of (A) Maximum likelihood phylogenetic tree derived by TPM2 + F + I + G4 model from available ITS1 sequences of anoplocephalid cestodes. Sequence of *Hymenolepis microstoma* (Accession number AJ287525) was used as an outgroup (not shown). (B) Maximum likelihood phylogenetic tree derived by TIM2 + F + R5 model from available *cox1* sequences (1127 bp) of anoplocephalid cestodes. The green circles mark nodes with ultrafast bootstrap and SH-like approximate likelihood ratio test higher than 75%. Size of the circle correlates to the value. Complete uncollapsed trees can be found in Supplementary files SF2 (ITS1) and SF3 (*cox1*). The trees were visualized and edited in iTOL v6 (Letunic and Bork, 2021). Sequences derived in this study are in blue boxes, sequences from GenBank are identified by accession number.

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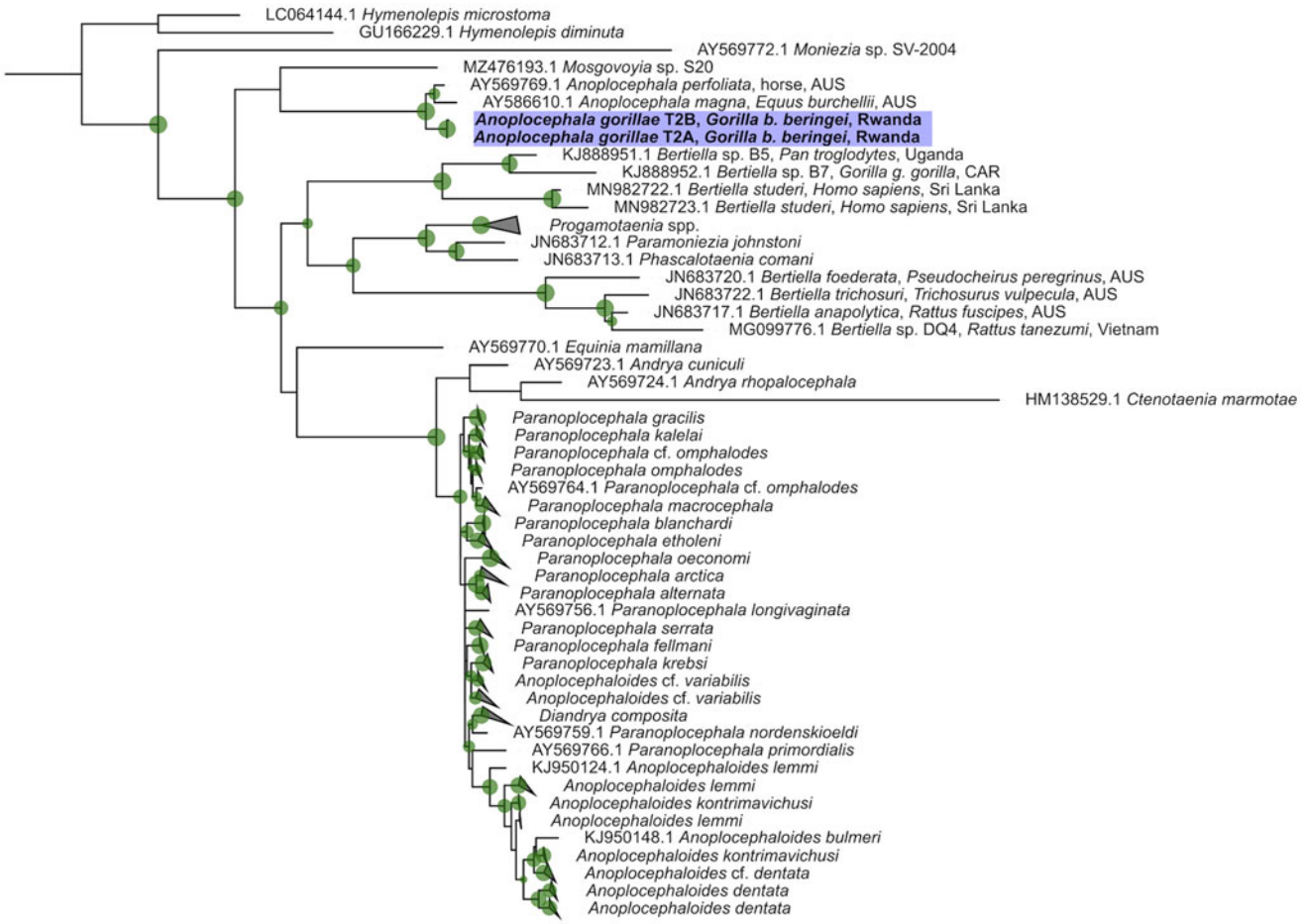


Figure 7. Maximum likelihood phylogenetic tree derived by TPM3u + F + R3 model from 1910 bp alignment of available 28S rDNA anoplocephalid cestode sequences. The green circles mark nodes with ultrafast bootstrap and SH-like approximate likelihood ratio test higher than 75%. Size of the circle correlates to the value. When appropriate, the clades have been collapsed (the uncollapsed tree can be found in Supplementary file SF4). The tree was visualized and edited in iTOL v6 (Letunic and Bork, 2021). Sequences derived in this study are in blue boxes, sequences from GenBank are identified by accession number. AUS, Australia; CAR, Central African Republic.

data as prevalence. Considering the results from coproscopy complemented by the PCR results, *A. gorillae* was detected in all 19 groups with percentage of positive samples ranging from 28.5% to 100% and reaching over 70% of positive samples in 15 out of 19 groups (Table 1).

During 2018–2019, in total 651 fecal samples were collected from 124 identified individuals belonging to 9 gorilla groups. One to eight samples per individual were obtained within 2 sampling periods (4 to 6 weeks). *Anoplocephala gorillae* prevalence in the groups ranged from 76.19 to 100%. The egg counts varied from 8 to 8190 EPG, with an average of 384 EPG and a median of 248 EPG. The youngest *A. gorilla*-positive individual was a 3-month-old infant from Hirwa group (8 EPG). The probability of detecting *A. gorillae*-positive individuals significantly increased with the number of samples examined (logistic regression; estimate = 1.49 ± 0.60, $F1124 = 36.8$, $P < 0.001$). The probability to detect a positive individual increased from 68% to 98% just by increasing the number of samples from 1 to 3. Most individuals (78.6%) were found *A. gorillae*-positive after examination of the first sample, but up to 4 samples were required to detect *A. gorillae* presence.

In most animals, a considerable fluctuation of EPG values over time was observed in samples collected from the same individual (Fig. 11A). In 9 gorillas, both positive and negative samples occurred within 1 sampling period. In 2 individuals, only 1 sample was collected and found negative by coproscopy, but the PCR showed presence of *A. gorillae* DNA (Fig. 11A).

In 35 samples (5.4%) originating from 22 individuals (17.6%), the EPG exceeded 1000 EPG. These outlying individuals comprised 10 males, 12 females, 13 adults, 2 subadults, 5 juveniles and 2 infants. In most of these animals, only a single sample had such high *A. gorillae* EPG, while in 8 individuals (3 adult males, 2 adult females and 3 juveniles/infants), high *A. gorillae* EPG values were recorded repeatedly. However, repeatability of high EPGs

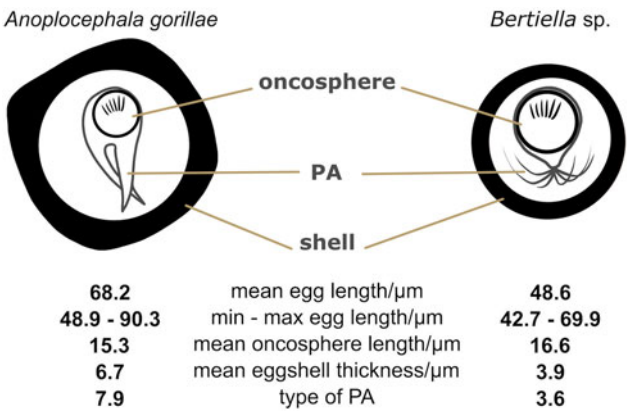


Figure 8. Comparison of *A. gorillae* and *Bertiella* sp. egg morphology. The type of pyriform apparatus (PA) was classified using a scale from 1 to 10, where 1 refers to clearly *Bertiella*-like PA (brush-like), whereas 10 refers to the *Anoplocephala*-like PA (bifurcated).

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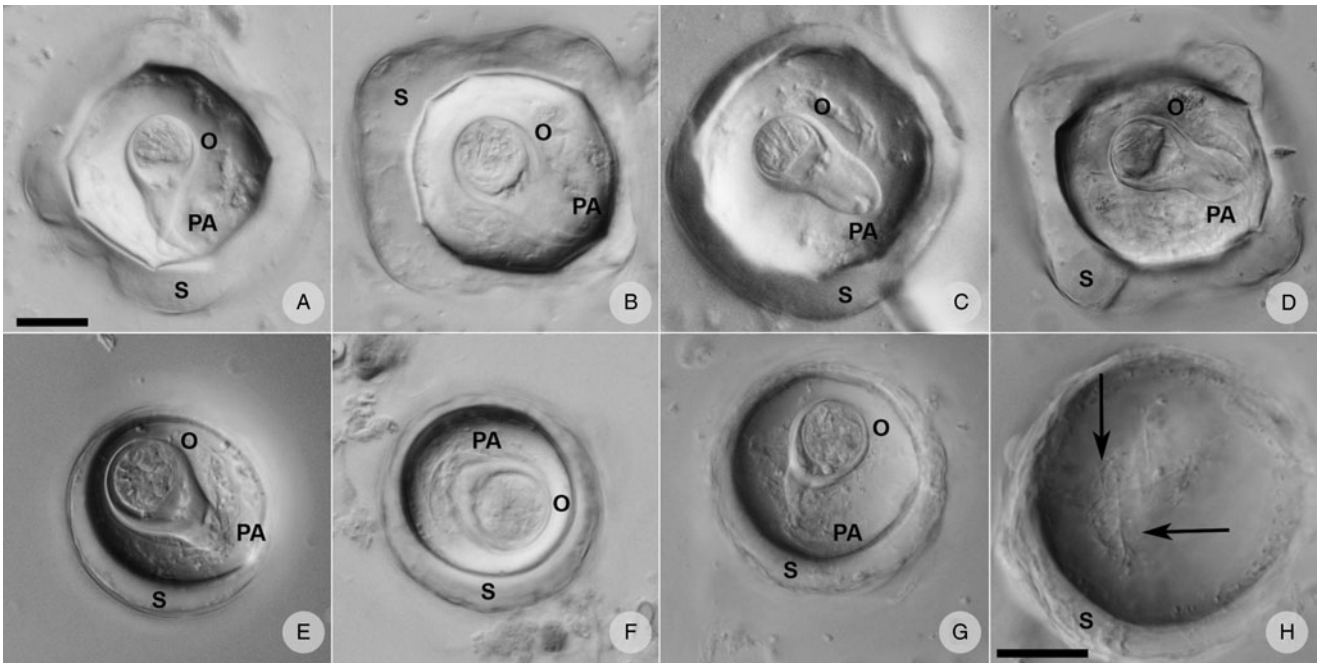


Figure 9. Anoplocephalid cestode eggs observed in mountain gorilla feces. (A—D) *Anoplocephala* morphotype typical for rectangular or polygonal shape, very thick shell (S), oncosphere (O) relatively smaller, and bifurcated pyriform apparatus (PA). (E—G) *Bertiella* morphotype characterized by round shape, thick shell, relatively bigger oncosphere and brush-like PA. (H) Detail of the PA fibres (arrows) visible when focusing throughout the depth of the egg. (A–G) on the same scale, both scales correspond to 20 μm.

for all individuals sampled more than once was generally low [r (95% CI) = 0.22 (0.14–0.30)].

Out of 124 individually sampled gorillas, 17 individuals from 10 different groups and between the age from 15 months and 20 years were *A. gorillae* negative. For the majority of these animals ($N = 13$), only 1 fecal sample was collected and examined. In 3 individuals (1 adult female and 2 infants), 2 samples were collected and examined. Finally, in 1 male juvenile all 3 samples were tested negative.

In 5 groups, sample sets from both, nest and individual sampling, were available from the same period allowing us to compare fecal prevalence (percentage of positive samples) and individual prevalence (percentage of positive individuals). In 3 groups, both prevalence values reached 100%, while in 2 groups, the individual prevalence was slightly higher than fecal prevalence (Fig. 11B).

Discussion

Using an extensive set of fecal samples, dozens of proglottids, and adult helminths, anoplocephalid cestodes parasitizing mountain gorillas in Rwanda were identified as *A. gorillae* and *Bertiella* sp. and characterized both morphologically and genetically. Specific PCR assays were provided for detection of both the dominant *A. gorillae* and the rarely occurring *Bertiella* sp. Prevalence and individual egg excretion trends of *A. gorillae* were described using fecal samples from identified individuals and nests.

Anoplocephalid cestode species are defined mainly by the morphology of proglottids and the position of genital organs (Beveridge, 1994). Although we did not examine histological sections of mature proglottids, all examined adults exhibited morphological features described by Nybelin (1927) as typical of *A. gorillae*, particularly the suckers, which are terminally attached

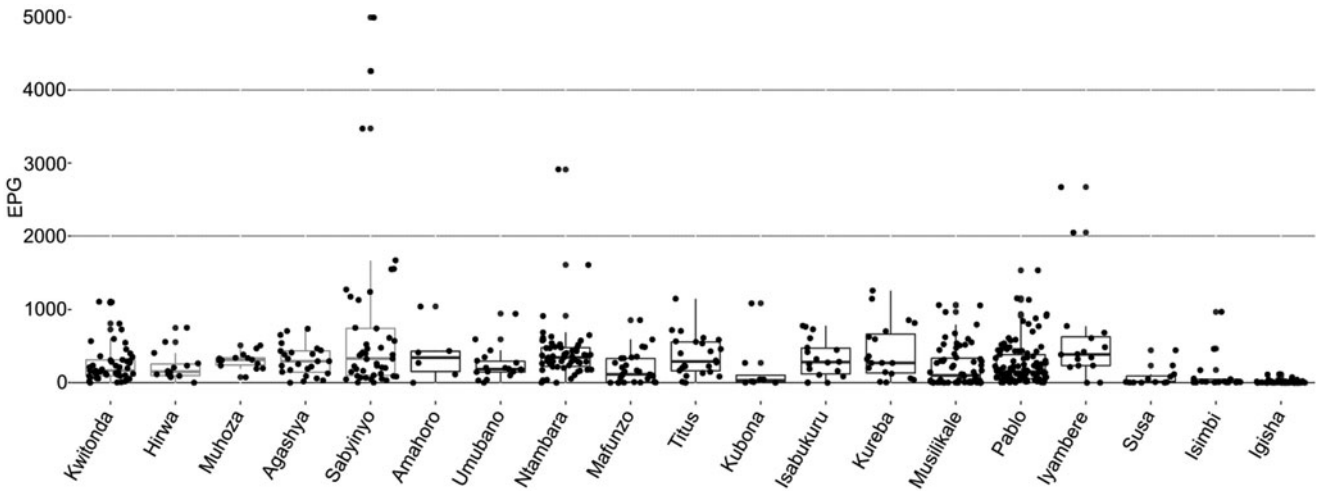


Figure 10. *Anoplocephala gorillae* egg counts in studied mountain gorilla groups expressed as eggs per gram of feces (EPG) including all samples collected between 2018 and 2019. *X* axis shows individual gorilla groups. For statistical analyses of EPG see Petrželková *et al.*, 2021.

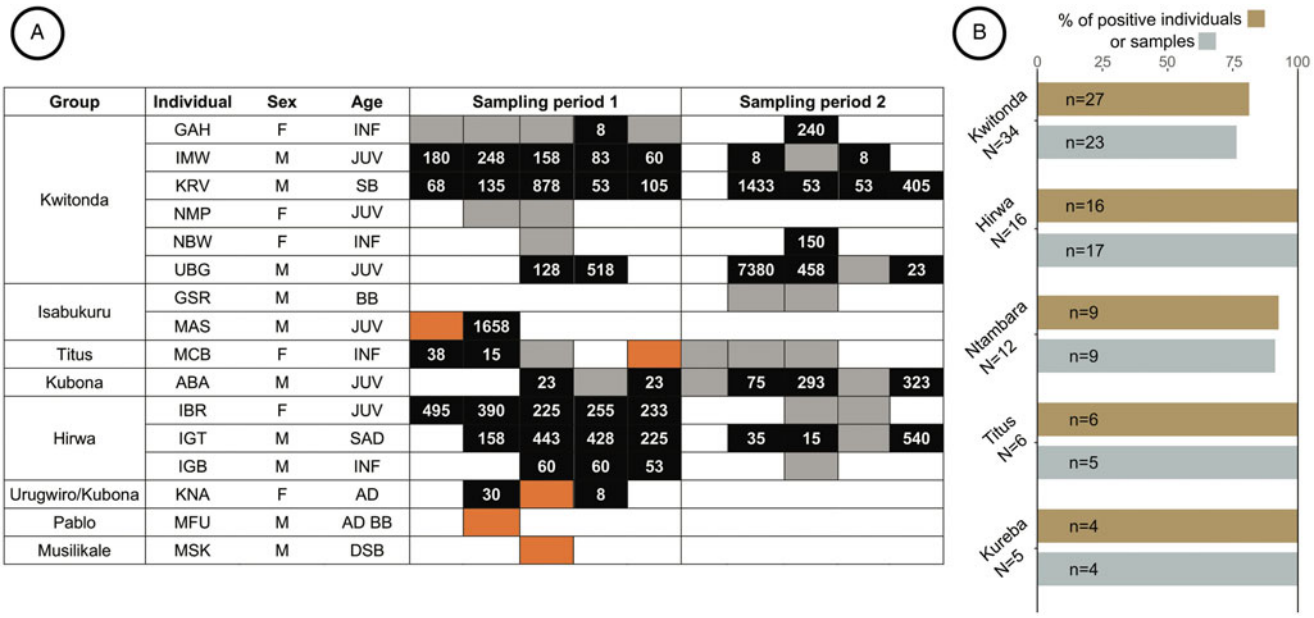
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Table 1. Percentage of *Anoplocephala gorillae*-positive nest samples (PAPS) in dry (Jan–Feb) and wet (Sep–Oct) seasons by groups in 2018

		Dry		Wet	
Group	Group size	No. of samples	PAPS (%)	No. of samples	PAPS (%)
Kwitonda	30	26	100	23	91.3
Hirwa	17	14	100	17	76.5
Muhoza	8	8	100	6	100
Agashya	14	14	100	11	90.9
Sabyinyo	17	10	100	15	100
Amahoro	19	NA	NA	6	100
Umubano	12	9	100	9	88.9
Ntambara	12	10	100	9	100
Mafunzo	14	NA	NA	11	81.8
Titus	6	6	100	5	100
Kubona	NA	NA	NA	5	80
Isabukuru	13	9	100	9	66.7
Kureba	8	7	100	4	100
Musilikale	18	11	100	12	66.7
Pablo	23	16	93.7	17	94.1
Iyambere	6	4	100	4	100
Susa	18	12	100	16	75
Isimbi	18	9	77.8	8	62.5
Igisha	26	16	62.5	14	28.5

Coproscopy results were corrected by PCR results (adding falsely negative samples). NA – no samples collected.

to a small scolex without any rostellum. Other *Anoplocephala* species have a similar gross morphology, however the DNA sequence analysis showed clear differentiation of *Anoplocephala* from mountain gorillas and *A. perfoliata*, *A. magna*, *A. rhodesiensis* from equids and *A. manubriata* from elephants. Since *A. gorillae* is the only species of the genus described from mountain gorillas, we concluded that the cestodes found in mountain gorilla necropsies are *A. gorillae*, as named by Nybelin (1927). The 18S rDNA



Group	Individual	Sex	Age	Sampling period 1					Sampling period 2			
Kwitonda	GAH	F	INF				8			240		
	IMW	M	JUV	180	248	158	83	60	8		8	
	KRV	M	SB	68	135	878	53	105	1433	53	53	405
	NMP	F	JUV									
	NBW	F	INF							150		
	UBG	M	JUV			128	518		7380	458		23
Isabukuru	GSR	M	BB									
	MAS	M	JUV		1658							
Titus	MCB	F	INF	38	15							
Kubona	ABA	M	JUV			23		23		75	293	323
Hirwa	IBR	F	JUV	495	390	225	255	233				
	IGT	M	SAD		158	443	428	225	35	15		540
	IGB	M	INF			60	60	53				
Urugwiro/Kubona	KNA	F	AD		30		8					
Pablo	MFU	M	AD BB									
Musilikale	MSK	M	DSB									

Figure 11. (A) Examples of *Anoplocephala gorillae* EPG fluctuation in selected individuals resampled during 2 sampling periods (May-July 2018 and September-November 2018). Black = *A. gorillae* positive sample with EPG value; grey = *A. gorillae* negative sample; white = animal not sampled; orange = microscopy negative, but PCR positive; M, male; F, female; INF, infant; JUV, juvenile; SAD, subadult; AD, adult; BB, blackback; SB, silverback; DSB, dominant silverback. (B) Comparison of information gained through individual sampling, providing individual prevalence (percentage of positive individuals) and nest sampling, providing percentage of positive samples (PPS). N = total number of individuals in the group, n = number of samples (nest sampling) or individuals examined

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and ITS1 sequences obtained from all proglottids shed in feces matched the reference sequences obtained from the identified adult cestodes, and in the vast majority of the fecal samples examined, we detected *A. gorillae* DNA. However, we also found 1 gorilla infant that harboured *A. gorillae* along with an undetermined *Bertiella* sp.

Bertiella is another anoplocephalid cestode genus that commonly parasitizes primates, including humans, but also rodents, marsupials and dermopterans (Beveridge, 1994; Wickström *et al.*, 2005; Beveridge *et al.*, 2007; Hardman *et al.*, 2012; Sapp and Bradbury, 2020) and apparently also mountain gorillas, although rarely. The only material available for the identification of this *Bertiella* found in a single mountain gorilla individual was the eggs. Anoplocephalid cestode eggs are typical for the presence of a PA, which encloses the oncosphere (Beveridge, 1994), and egg morphology can be used to distinguish many of the genera within the family (e.g. Conn, 1985; Beveridge, 1985*b*; Galán-Puchades *et al.*, 2000; Haukisalmi and Henttonen, 2000; Tenora *et al.*, 2002), sometimes even species (Bohórquez *et al.*, 2014). Detailed morphometric analysis of the 2 anoplocephalid cestode species detected in the Virunga mountain gorillas revealed that *A. gorillae* can be reliably distinguished from *Bertiella* sp. by a polymorphic shape ranging from triangular to polygonal, a larger absolute size reaching up to 90 μm, proportionally smaller oncosphere, and PA clearly bifurcated into only 2 branches. In contrast, *Bertiella* eggs are typically spherical or subspherical. Due to the smaller size of the egg, the oncosphere occupies a larger area of the egg and eventually, the PA branches into numerous fine, wavy filaments. It should be noted that the filamentous character of PA is typical for *Bertiella* spp. in primates, while in other hosts, such as marsupials, both filamentous as well as paired PA occur commonly (Beveridge, 1985*a*). The presence of *Bertiella* in our sample set would not have been noticed without discrepancies in PCR results, demonstrating the need for personnel conducting the microscopy to be properly trained and experienced. In an ideal case, simultaneous DNA-based diagnostic techniques should be performed.

Although a number of *Bertiella* spp. sequences are available in GenBank, none of them showed conspecificity with our mountain gorilla isolate, and we are not able to assign our *Bertiella* sp. isolate to any sequenced species. Surprisingly, analyses of the nuclear ITS1 sequence showed that our *Bertiella* from Virunga gorillas is genetically closer to the South American isolates (about 11% difference) than to the Ugandan isolates (more than 30% difference), which is in agreement with the results of Doležalová *et al.* (2015) who showed a close relationship between isolates from Peru, Brazil and Kenya with an overall genetic distance of 0.2–12% based on *cox1*. Unfortunately, we were not able to obtain *cox1* sequences from our sample and could not compare the results with the latter study.

While 29 species of *Bertiella* have been described (Beveridge, 1994), sequences from only 3 primate-associated species (*B. mucronata*, *B. satyri*, *B. studeri*) and 4 other species from rodents and marsupials (*B. affinis*, *B. anapolytica*, *B. foederata* and *B. trichosuri*) are available in GenBank. More than half of the *Bertiella* spp. sequences available in database belong to undetermined species, mostly from primates. Such a lack of sequences may impact the topology of phylogenetic trees in our ITS1 analysis. While multiple markers have been sequenced for *Bertiella* spp. from primates, only 28S rDNA sequences of *Bertiella* spp. from rodents and marsupials have been obtained. The sequences of additional markers are necessary to confirm the polyphyly of the genus *Bertiella* as seen in the 28S rDNA phylogenetic tree, where the sequences from primate parasites form clades separated from *Bertiella* spp. parasitizing rodents and marsupials by clades of *Progamotaenia* spp., *Paramoniezia johnstoni* and *Phascalotaenia comani*. This polyphyly would support separation of the *Bertiella* spp. parasitizing in primates from species occurring in marsupials, rodents and dermopterans as suggested by Baer (1927) who suggested the genus *Prototaenia* for the latter group, based mainly on the level of cirrus sac development and fine differences of other reproductive organs. These distinctions were subsequently deemed as insufficient for classification of a separate genus by Baer (1934) and thus, single genus *Bertiella* has been accepted ever since (Beveridge, 1994). Additional sequences from properly determined *Bertiella* species will help clarify the phylogeography and taxonomy of the genus.

The study by Doležalová *et al.* (2015) also published a sequence of *Anoplocephala* cf. *gorillae* (isolated from eggs in a single fecal sample), but the sequence clustered within the *Bertiella* clade. The authors suggested that *A. gorillae* should be moved to the genus *Bertiella*. In their study, sequences of 5.8S-ITS2 region (JQ771100), *nad1* (JQ771117) and 18S rDNA (MW741845) were obtained from this particular cestode referred to as *A. gorillae* and both ITS2 region and 18S rDNA differed significantly from our *A. gorillae* sequences (60.3% and 18% respectively). Moreover, our *Bertiella* 18S rDNA sequence clustered in a different subclade than the cestode from a mountain gorilla sequenced by Doležalová *et al.* (2015) (Supplementary file SF1) and the pairwise sequence distance between the 2 sequences was 2.1%. Considering that our results are based on a solid sample set and that the previous study used only 1 fecal sample as DNA source, we can conclude that *A. gorillae* indeed is a member of *Anoplocephala* and that it was presumably another *Bertiella* sp. that was sequenced in the previous study. As the *Bertiella* spp. seemed to be rather rare, the mountain gorillas are most probably infected by an occasional spill-over from different hosts, as observed in *Bertiella* and other species within the Anoplocephalinae (Beveridge, 1994; Haukisalmi *et al.*, 2004, 2008, 2009; Wickström *et al.*, 2005; Hardman *et al.*, 2012).

Microscopy following flotation or sedimentation techniques represents the most common approach to routine coproscopic detection of metazoan gastrointestinal parasites in wildlife (Masi *et al.*, 2012; Vimalraj and Jayathangaraj, 2015; Naz *et al.*, 2021; Petrželková *et al.*, 2021) due to its cost effectiveness and simplicity. However, sensitivity of these methods and challenging identification of detected parasites commonly lead to biased results. In our sample set, all fecal samples scored as negative using Mini-FLOTAC© were re-examined by sedimentation and PCR, which combined detected almost 20% of these samples containing either *A. gorillae* eggs or DNA. Egg numbers determined *via* sedimentation in these falsely negative samples ranged from very low (5 EPGS) to very high, reaching over 2000 EPGS. Such discrepancy may be caused partially by slightly higher sensitivity of the sedimentation method, which was 5 EPGS in our case, while the threshold for Mini-FLOTAC© was 8 EPG. On the other hand, it is not surprising that PCR detected falsely negative samples from microscopy, as eggs might be shed irregularly, while the parasite DNA is usually present in the amount sufficient for molecular detection (Wong *et al.*, 2014; Rijsman *et al.*, 2016) Nevertheless, in 1 case, we were not able to amplify *A. gorillae* DNA using PCR despite the eggs observed in sedimentation, though in low numbers (5 EPGS). The falsely negative samples represented only 1.3% of the total sample set and 1.5% of samples from identified individuals and, therefore, their effect on overall results is marginal. However, 2 false-negative samples were the only samples collected from 2 gorilla individuals and therefore, without PCR, these 2 gorilla males would be considered as *A. gorillae* negative.

Even though there are apparently 2 anoplocephalid cestodes parasitizing Rwandan mountain gorillas, *Bertiella* sp. was detected in a single individual, moreover in co-infection with *A. gorillae*.

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Thus, the epidemiological and ecological data of this mountain gorilla subpopulation can be related to *A. gorillae*. This cestode is a common symbiont in the intestine of mountain gorillas a few months after birth. Previous studies also reported a high percentage of cestode-positive fecal samples in both Virunga and Bwindi populations (Redmond, 1983; Ashford *et al.*, 1996; Sleeman *et al.*, 2000; Kalema-Zikusoka *et al.*, 2005). This situation contrasts strongly with other African great apes where cestode infections are only rarely detected (Ashford *et al.*, 2000; Lilly *et al.*, 2002; Krief *et al.*, 2005; Kooriyama *et al.*, 2012; McLennan and Huffman, 2012; Sá *et al.*, 2013; Drakulovski *et al.*, 2014; Pafčo *et al.*, 2017; Vlčková *et al.*, 2018) even though seasonal peaks of infections were observed (Wrangham, 1995). Occasional infections are reported in orangutans (Mul *et al.*, 2007), *Colobus* spp. (Gillespie *et al.*, 2005), and other non-human primates, such as red guenon (*Cercopithecus ascanius*; Gillespie *et al.*, 2004), baboons (Kooriyama *et al.*, 2012), Verreaux's sifaka (*Propithecus verreauxi;* Rasambainarivo *et al.*, 2014) and blue monkeys (*Cercopithecus mitis*; Appleton *et al.*, 1994).

Muhangi *et al.* (2021) reported anoplocephalid cestodes in about a third of 60 mountain gorillas necropsied between 1985 and 2007 in both Virunga and Bwindi and even though simultaneous presence of cestodes and haemorrhagic and/or hyperaemic mucosa of the small intestine (8/60 individuals) and villous blunting (2/60 individuals) were detected, no lesions were observed in 14 animals with cestodiasis. Moreover, during necropsies performed on mountain gorilla individuals deceased e.g., from trauma and generally in good condition, large number of tapeworms were found in the intestines (GD, *personal observation*). It appears that unlike *A. perfoliata,* which is strongly associated with colic in horses (Mezerova *et al.*, 2007; Back *et al.*, 2013), *A. gorillae* does not have significantly negative health effects on its host.

In all anoplocephalid cestodes, gravid proglottids detach from an adult (Redmond, 1983; Pafčo, 2018) and are passed through the intestines with the content. Based on the species, proglottids can be shed intact, or disintegrate during their way through the gastrointestinal tract releasing the eggs. Redmond (1983) reported that *A. gorillae* eggs remain in proglottids, however, we did not observe any eggs in any of the 5 proglottids examined. The almost ubiquitous presence of *A. gorillae* eggs in feces suggests that the eggs are released from the proglottids into the intestinal content, similarly to some other anoplocephalid species (Schuster, 1991; Deplazes *et al.*, 2016). As a result, coproscopy is a sufficient method for the detection of cestodes in mountain gorillas unlike *A. perfoliata* in horses (Traversa *et al.*, 2008; Tomczuk *et al.*, 2014). Traditionally, the egg quantities (expressed as EPG) are used as a proxy of infection intensity in helminths parasitizing both domestic animals and wildlife (Williamson *et al.*, 1998; Chapman *et al.*, 2006; Tomczuk *et al.*, 2014; Burgunder *et al.*, 2017). Individuals with high EPG would expectedly be at higher risk of clinical disease and, due to an uneven distribution of macroparasites commonly found within host populations (Shaw *et al.*, 1998; Wilson *et al.*, 2001), be also the main reservoir and source of infection for other individuals when shedding most eggs to the environment. From the population management point of view, such animals would be the best targets for interventions such as anthelminthic treatment (Nielsen *et al.*, 2019). Ashford *et al.* (1996) reported that mountain gorilla silverbacks in Bwindi shed most cestode eggs, while infants shed no eggs or only a low number of eggs. In our previous study (Petrželková *et al.*, 2021), infants in the Virunga population also shed significantly fewer eggs than all other age categories, but that was the only significant trend applicable at the population level. In certain groups, a peak of infection was detected between 10 and 20 years of age and similarly, relationship between group size and egg counts was observed in some groups and/or areas (Petrželková *et al.*, 2021). Closer inspection of *A. gorillae* egg shedding patterns in this study revealed a significant EPG fluctuation in individual mountain gorillas within short time periods. Even animals with extreme EPG values (defined in our case as more than 1000 EPG) usually had only 1 sample with such high EPG value. Among 8 individuals with repeatedly high EPG, all age categories as well as both sexes were represented, suggesting that there is no obvious trend regarding the sex or age, but as the number of such individuals was low, we did not test the hypothesis by statistical methods. Egg shedding fluctuation greatly complicates using EPG as a proxy for infection intensity in *A. gorillae*, and, if only a single value is considered for an individual, it may be largely misleading.

Wildlife parasitology, especially in cases of endangered and/or elusive species, relies on noninvasively collected samples. In our case, the *A. gorillae* prevalence in unidentified nest fecal samples and in samples from identified individuals from the same group showed comparable results, which has implication for the gorillas' health monitoring. One-time examination of easily accessible unidentified nest samples provides reliable information about helminth prevalence in the given group. However, multiple sampling of individuals confirmed that even though the cestodes commonly occur in mountain gorillas, the number of gorilla individuals detected as *A. gorillae*-positive further increases with the number of samples tested. It has been suggested previously that at least 3 samples should be examined from an individual primate to reliably detect the spectrum of parasites infecting given individual and to determine realistically the parasite prevalence in a primate group or population (Huffman *et al.*, 1997; Muehlenbein, 2005). Our results confirm that such a minimum of samples is needed also for assessment of a parasite as prevalent as *A. gorillae*.

In conclusion, the adult cestodes collected during necropsies helped us establish the identity of *A. gorillae* and provide reference sequences essential for phylogenetic analyses as well as for the development of advanced diagnostic tools. We were unable to associate observed high abundance of *Anoplocephala* with any pathologies, suggesting that the anoplocephalid infection does not represent a threat for the studied subpopulation of *G. beringei beringei*.

Supplementary material. The supplementary material for this article can be found at https://doi.org/10.1017/S0031182023001178.

Data availability statement. All sequences produced in this study are deposited in the GenBank under accession numbers OR863079-91, OR863669-80 and OR864671-76. A collection of *A. gorillae* adult specimens used in this study was deposited to the Museum of Environment, Karongi District, Rwanda. Contact: fdushimana@rwandaheritage.gov.rw

Acknowledgements. We are thankful to the Rwanda Development Board for their long-term support and permission to conduct this research. We are indebted to the field staff of the Dian Fossey Gorilla Fund, Rwanda Development Board and Volcanoes National Park for their tireless support in collecting fecal samples.

Author's contributions. KJP, MC, KG and DM obtained funding. KJP, MC, KG, WE, BP, BČ and DM designed the study. MC, KG, JBN, JN, WE, FN, AM, RM and PU organized and supervised the fecal sample collection. JŠ, DM, BČ and BP designed and optimized field laboratory work. MC, KG, JN, JBN, GN, AEN and MB performed autopsies, collected adult cestodes and provided clinical information. CU, MB, BČ, BP, TP and RMC processed the samples in the field laboratory and performed the coproscopic examinations. TP and BČ analysed the cestode morphology. TP and RMC performed the nucleic acid amplification tests. BČ supervised the laboratory analyses, analysed the DNA sequencing data and performed phylogenetic analyses. TP created the line artwork with the egg morphology and workflow. BČ, TP, DM and MC provided photographs. PS performed statistical analyses. DR produced the map of the study area. TP prepared the first draft of the manuscript. BČ finalized the

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manuscript and implemented comments and suggestions from all co-authors. All authors contributed to the final version of the manuscript and approved it.

Financial support. The parasitological part of this study was supported by the Czech Science Foundation (18-24345S) and the U.S. Fish and Wildlife Service Division of International Affairs, Grant no. F17AP00964. Klára Petrželková and Barbora Pafčo were also supported by institutional support of the Institute of Vertebrate Biology, Czech Academy of Sciences (RVO:68081766).

Competing interests. None.

Ethical standards. Material used in the study comprised fecal samples non-invasively collected from night nests or during daily follows of studied animals following the *Best practice guidelines for health monitoring and disease control in great ape populations* (Gilardi *et al.*, 2015). The studied gorillas are habituated to human presence and collection of fecal samples does not induce stress to them. Autopsies of deceased animals are routinely performed by GD as part of the mountain gorilla health monitoring.

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Uwamahoro, C., Holubová, N., Jirků, M., Sak, B., Květoňová, D., Samaš, P., Červená, B., Romportl, D., Eckardt, W., Ndagijimana, F., Muvunyi, R., Uwingeli, P., Mapilanga, J., Kalonji, A., Aruho, R., Cranfield, M., Gilardi, K., Nziza, J., Ssebide, B., Kambale Syaluha, E., Pafčo, B., Jirků, K., Kváč, M., Petrželková, K.J.

2025

Cryptosporidium, microsporidia and _Giardia_ infections in endangered mountain gorillas inhabiting Virunga Massif.

J. Vertebr. Biol. 74, 25035. https://doi.org/10.25225/jvb.25035

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J. Vertebr. Biol. 2025, 74: 25035 DOI: 10.25225/jvb.25035

RESEARCH PAPER

Cryptosporidium, Microsporidia and *Giardia* infections in endangered mountain gorillas inhabiting Virunga Massif

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Received 25 April 2025; Accepted 9 July 2025; Published online 23 September 2025

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Abstract. The number of mountain gorillas (*Gorilla beringei beringei*) has grown steadily in recent decades thanks to intensive conservation efforts. But as they live in restricted areas, they experience changes in population dynamics and pathogen epidemiology. These changes have been demonstrated by the emergence of gastrointestinal problems linked to strongylid infections in the Virunga population, highlighting the need to study changes in other parasite infections. We assessed the patterns of *Cryptosporidium*, *Giardia* and microsporidia infections in mountain gorillas across the Virunga Massif. We examined 152 faecal samples from 16 gorilla groups collected in 2018 using PCR and qPCR on three genetic markers and detected *Cryptosporidium muris* (< 1%), *Enterocytozoon bieneusi* (genotypes-D 14%, CHN-F1 < 1%) and *Giardia intestinalis* (13%). No significant spatial or sex/age differences were observed in microsporidia and *Giardia* infections. However, a temporal comparison with previous data suggests changes in the protist communities over time. We conclude that these protists were not impacted by the changes in mountain gorilla population dynamics and did not contribute to the increasing occurrence of severe gastrointestinal problems in Virunga gorillas. These results aid our epidemiological understanding of parasite communities in mountain gorillas, an asset for their ongoing conservation management in the face of newly emerging health challenges.

Key words: epidemiology, PCR, protist, *Encephalitozoon*, *Enterocytozoon*, non-human primate

Introduction

Mountain gorillas are endemic to the Virunga Massif and Bwindi – Sarambwe ecosystem. In 1960, the Virunga gorilla population size was estimated to be between 400 and 500 individuals, which was reduced to between 260 and 290 in 1973 due to human-induced threats including hunting, habitat destruction and cattle disturbance, among others (Weber & Vedder 1983). Thanks to a wide range of continuing conservation actions, including daily group monitoring and protection, veterinary interventions, anti-poaching measures and community-driven conservation, the population in the Virunga Massif has increased significantly in the last approximately 40 years, largely due to the growth in habituated groups (Robbins et al. 2011, Hickey et al. 2019a). The total mountain gorilla population now exceeds 1,000 individuals when combined with the Bwindi – Sarambwe population (Hickey et al. 2019a, b). This recovery led to the downgrade of their IUCN status from Critically Endangered to Endangered (Hickey et al. 2018).

However, the potential for further spatial expansion of mountain gorilla habitat is extremely limited due to the dense human communities adjacent to their forest refuges (Caillaud et al. 2014, 2020). The growing gorilla populations living in these restricted areas may experience changes in stress levels and pathogen epidemiology, affecting their health (Caillaud et al. 2014, Eckardt et al. 2019, Petrželková et al. 2021). Moreover, whole genome sequencing indicated extensive inbreeding in mountain gorillas, with the loss of genetic diversity appearing to have started over 20,000 years ago, potentially caused by changes in climate and human-associated effects (Xue et al. 2016). With low genetic diversity, mountain gorillas can be particularly sensitive to pathogens as heterozygosity levels are linked directly to reduced population fitness via inbreeding depression (Reed & Frankham 2003).

The population increase in the Virunga Massif has not been uniform and is likely influenced by varying ecological conditions, food availability, and level of human disturbances (Kalpers et al. 2003, Ganas & Robbins 2005, Gray et al. 2013). In recent years, the gorilla subpopulation in the area between Mt. Karisimbi and Mt. Bisoke (aka Visoke) increased in size by almost 50% and group density as well as home range overlap increased (Caillaud et al. 2014, 2020). Subsequently, changes in the social behaviour were observed, e.g. an increase in intergroup encounters and male-male aggression, and a fivefold increase in the rate of infanticide (Caillaud et al. 2020). Observed intergroup encounters are a significant source of stress, with increased faecal glucocorticoid metabolite levels up by almost nine times compared to baseline levels (Eckardt et al. 2016, 2019), and this could influence susceptibility to pathogens (Petrželková et al. 2022).

Gastrointestinal problems, namely chronic gastritis and chronic wasting syndrome, linked to helminth infections have increasingly affected both mountain gorilla populations in recent years, posing a significant threat to these endangered animals (Muhangi et al. 2021, Gorilla Doctors unpublished data). To date, striking geographic differences in

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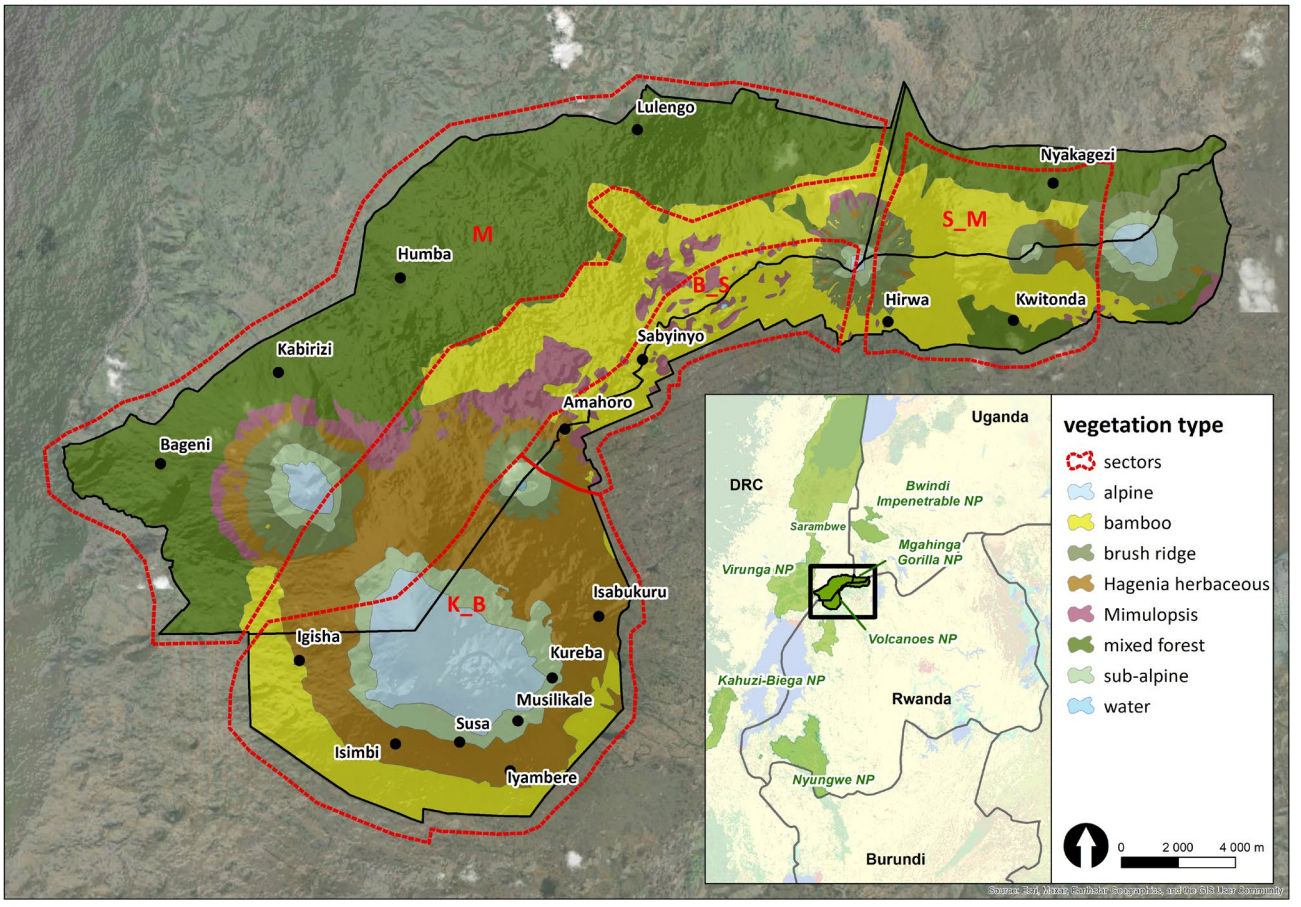


Fig. 1. Division of the Virunga Massif into four areas according to volcano positions with location of the study groups. M: Mikeno; K_B: Karisimbi-Bisoke; B_S: Bisoke-Sabyinyo; S_M: Sabyinyo-Muhabura). The map is based on Fig. 1 from Petrželková et al. (2021) and was created by using ArcGIS Desktop 10.6 (ESRI 2019. ArcGIS Desktop: Release 10.6. Redlands, CA: Environmental Systems Research Institute; esri.com). Vegetation data were adopted according to Robbins et al. (2011), and boundaries of protected areas were derived from the ProtectedPlanet.net database.

gastrointestinal strongylid infections within the Virunga population have been revealed with higher strongylid egg counts (Petrželková et al. 2021, 2022) and higher absolute abundance of pathogenic gastric strongylid taxa in the area with high occurrences of gastritis, namely in the area between Mt. Karisimbi and Mt. Bisoke (Mason et al. 2025). These results have pointed to new challenges emerging as possible 'side effects' of the remarkable conservation success of the past few decades, and call for further examination of the patterns of infection of other mountain gorilla parasites.

This study focuses on gastrointestinal protists in the Virunga Mountain gorilla population, specifically microsporidia, *Giardia intestinalis* and *Cryptosporidium* spp. These protists are transmitted through the faecal-oral route via environmentally resistant stages and can infect humans, livestock and wildlife, including non-human primates (Nizeyi et al. 1999, Sak et al. 2011c, Modrý et al. 2018). Limited research has been conducted on the molecular diversity and epidemiology of these opportunistic pathogens in Virunga gorillas, with existing studies reporting the presence and diversity of these parasites in this population (Hogan et al. 2014, Sak et al. 2014).

We conducted molecular screening for *Cryptosporidium*, *Giardia*, and microsporidia infections in faecal samples collected from mountain gorillas across the entire range of their habitat in the Virunga Massif. This region spans 451 km² and includes Volcanoes National Park in Rwanda, the Mikeno sector of Virunga National Park in the Democratic Republic of the Congo (DRC), and Mgahinga Gorilla National Park in Uganda (Fig. 1). We used both conventional PCR and the more sensitive qPCR, targeting various genetic markers. To assess temporal changes in these protist infections over an approximately ten-year period, we compared our results to those of Sak et al. (2014), who analysed the same population but focused on the subpopulation in the Rwandan section of the Virunga Massif using samples collected in 2007 and a PCR-only approach. As we implemented an additional, more sensitive method and surveyed the whole Virunga Massif, we predicted we would find

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a higher prevalence and a higher diversity of those protists in the studied samples compared to the 2007 samples. We also explored geographical differences in the occurrence of *Cryptosporidium*, microsporidia and *Giardia* infections across Virunga Massif and predicted a higher occurrence of these pathogens in the area between Mt. Karisimbi and Mt. Bisoke compared to other areas, mirroring spatial strongylid infection patterns (Petrželková et al. 2021). Last, we evaluated the impact of sex and age on these protist infections. We predicted that the prevalence of *Cryptosporidium* and *Giardia* decreases with increasing age due to repeated exposures to infection and the development of protective immunity (Checkley et al. 1997, Maddox-Hyttel et al. 2006, Kotloff et al. 2013). In contrast, we predicted that older animals are more frequently parasitised by microsporidia as reported in the human population due to the cumulative load and limited effect of the immune response to control microsporidia infection (Sak et al. 2011a, 2017). Like in other animal species and humans, we do not assume that sex influences the occurrence of the protists studied (Chen et al. 2002, Hunter & Nichols 2002, Sak et al. 2011b).

Material and Methods

Study area description

The Virunga Massif encompasses a diverse montane ecosystem ranging from 2,000 to 4,500 m a.s.l. and includes a mosaic of vegetation types shaped primarily by elevation. These range from alpine zone (above 3,600 m a.s.l.) and sub-alpine zone (3,200-3,600 m a.s.l.), to mid-elevation habitats such as *Hagenia* – *Hypericum* zone (2,800-3,200 m a.s.l.), bamboo zone (2,500-2,800 m a.s.l.) and lower altitude forest (mixed forest) (1,600-2,500 m a.s.l.), as well as disturbed woodland (2,300-2,800 m a.s.l.), open grassland and swamp (Watts 1998, Owiunji et al. 2005). The area experiences two rainy seasons (March-May and September-November) and two dry seasons (December-February and June-August).

We divided the Virunga Massif into four study areas based on volcano positions: Karisimbi-Bisoke (K_B): Volcanoes NP; Bisoke-Sabyinyo (B_S): Volcanoes NP; Sabyinyo-Muhabura (S_M): Volcanoes NP, and Mgahinga Gorilla NP, Mikeno (M): Virunga NP. This division follows previous ecological stratifications used in the helminth infection study of Petrželková et al. (2021). These spatial sectors serve as proxies for variation in vegetation types, patterns of historical subpopulation growth, and gastrointestinal disease occurrence. For example, the Karisimbi-Bisoke sector has experienced a disproportionate increase in group density and stressful intergroup encounters. (Caillaud et al. 2014, 2020, Eckardt et al. 2019) and higher prevalence of strongylid-associated gastritis (Petrželková et al. 2021, 2022). By adopting this ecologically grounded division, we aimed to assess whether spatial patterns in protist infections mirrored those previously observed in pathogenic strongylid infections (Petrželková et al. 2021, 2022).

Sample collection

In September and October 2018, 152 faecal samples were non-invasively collected from 16 monitored gorilla groups in the Virunga Massif (Fig. 1) by the Dian Fossey Gorilla Fund, Mountain Gorilla Veterinary Project (Gorilla Doctors), Rwanda Development Board, Institut Congolais pour la Conservation de la Nature, and Uganda Wildlife Authority staff. This collection represents approximately 39% of all identified social groups in the Virunga Massif at the time of the last census (Hickey et al. 2019a). The samples were obtained in the morning from gorilla nests of the previous night, labelled with the group name, collection date, and estimated age/sex class of the individual, inferred from faecal lobe diameter following established thresholds: silverback males (> 12 years, ≥ 7.3 cm), adult females, blackback males and subadults (> 6 years, > 5.5-7.2 cm), juveniles (3.5-6 years, 4.1-5.5 cm), and infants (0-3.5 years, ≤ 4.0 cm) (Petrželková et al. 2021, Sinayitutse et al. 2021). The groups were assigned to particular areas in Virunga (see above for division) based on GPS points recorded daily by staff of Dian Fossey Gorilla Fund, Gorilla Doctors, Rwanda Development Board, Virunga National Park, and Uganda Wildlife Authority in 2018. Samples were initially stored in refrigerators at laboratories of Gorilla Doctors in Rwanda, Uganda, and the DRC, then preserved in 96% ethanol and stored at –20 °C. Finally, the samples were transported to the Institute of Vertebrate Biology of the Czech Academy of Sciences, Czech Republic, for analyses, where they were kept at –20 °C.

Protist genotyping

The suspension of each faecal sample in 96% ethanol was evaporated overnight at 60 °C. Total genomic DNA (gDNA) was extracted from 200 mg of faeces using GeneAll® Exgene™ Stool DNA Mini Kit (GeneAll Biotechnology Co., Ltd.; Seoul, South Korea) and stored at –20 °C before amplification by PCR. Bead disruption of oocysts/cysts/spores for 60 s at 5.5 m/s using 0.5 mm glass beads in a FastPrep®-24 Instrument (MP Biomedicals, CA, USA) was performed prior to DNA extraction. Molecular detection of specific

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DNA of screened parasites was based on the nested polymerase chain reaction (PCR) protocols for the amplification of the chosen genes of *Cryptosporidium* spp. (18S rDNA), *Enterocytozoon bieneusi* (ITS-Internal Transcribed Spacer), *Encephalitozoon* spp. (ITS) and *G. intestinalis* (TPI-Triosephosphate Isomerase) (Katzwinkel-Wladarsch et al. 1996, Xiao et al. 1999, Sulaiman et al. 2003a).

For the 18S rDNA, ITS and TPI fragments, the primary PCR mixtures contained 2 µl of template gDNA, 2.5 U of Taq DNA Polymerase (DreamTaq Green DNA Polymerase, Thermofisher Scientific, Waltham, MA, USA), 1 × PCR buffer, 3 mM $MgCl_2$, 200 µM each deoxynucleotide triphosphate, 100 mM each primer and in 30 µl reaction volume. The secondary PCR mixtures were similar to those described above for the primary PCR, with the exception that 2 µl of the primary PCR product was used as the template DNA. DNA of *C. serpentis*, *E. bieneusi* genotype PtEbIX, *E. hellem* genotype 2B and *G. intestinalis* assemblage F and molecular grade water were used as positive and negative controls, respectively. The secondary PCR products were separated by electrophoresis on a 2.0% agarose gel and visualised following staining with ethidium bromide. Amplicons were purified using the GenElute™ Gel Extraction Kit (Sigma-Aldrich, St. Louis, MO, USA) and sequenced in both directions using the secondary PCR primers at a commercial laboratory (SEQme, Dobříš, Czech Republic). Sanger sequencing chromatogram files were edited using Chromas Pro 2.1.10 software (Technelysium, Pty, Ltd., South Brisbane, Australia), and sequences were verified by BLAST analysis (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The sequences obtained in this study and reference sequences obtained from GenBank were aligned using the software MAFFT version 7 online server (http://mafft.cbrc.jp/alignment/server/). The alignments were manually trimmed and edited in BioEdit v.7.0.5 (Hall 1999). Phylogenetic analyses were performed using the maximum likelihood (ML) method, using evolutionary models selected by MEGAX (Kumar et al. 2018). Bootstrap supports were calculated from 1,000 replications. Phylogenetic trees were produced by MEGAX and further edited for visualisation purposes with Corel DrawX7 (Corel Corporation, Ottawa, Ontario, Canada). Sequences have been deposited in GenBank under the accession numbers PQ867185 (18S rDNA), PQ860824-PQ860825 (ITS).

Quantitative PCR (qPCR)

Parasite load was expressed as the number of *Cryptosporidium* spp. oocysts (OPG), *E. bieneusi* and *Encephalitozoon* spp. spores (SPG) and *G. intestinalis* cysts (CPG) per gram of the faeces. For quantification of *Cryptosporidium* spp. and *E. bieneusi*, the commercial qPCR kit YSL-qP-EC-Crypto-100 and qPCR kit YSL-qP-IC-E.biene-100, respectively, were used according to manufacturer recommendations (YouSeq Ltd., Winchester, UK). Both kits contained positional controls. *Encephalitozoon* spp. was quantified using a TaqMan-based qPCR assay and previously published protocols (Wolk et al. 2002, Brožová et al. 2023). Briefly, each 25 µl reaction contained 2 µl gDNA, 400 nM forward and reverse primers, 200 nM 5′ FAM and 3′ BHQ1-labelled TaqMan probe, and 12.5 µl FastStart Universal Probe Master (Roche, Basel, Switzerland). For *Giardia*, we applied the qPCR diagnostic protocol utilising specific primers and a TaqMan probe labelled with FAM fluorescein (for details see Brožová et al. 2023) and used the Master Mix 5 × HOT FIREPol® Probe qPCR Mix Plus ROX (Solis BioDyne, Estonia). Positive control DNA was obtained from *Encephalitozoon cuniculi* genotype III and a *G. intestinalis* trophozoite cultures (WB ATCC 30957, human isolate).

Samples were run in triplicate, and Ct (Cycle threshold) values for each sample were recalculated based on the average reaction efficiencies, which were obtained upon comparative quantitation analysis. The standard curve and the negative control (DNA/DNAse/RNAse-free sterile water) were included in each analysis. Standard curves for *Cryptosporidium* and *E. bieneusi* were included in the kit, and for *Encephalitozoon* and *Giardia*, they were derived from serial dilutions of spore/trophozoite/gDNA (10^1-10^6). An internal amplification control (IAC) supplied in *Cryptosporidium* and *E. bieneusi* qRT-PCR kits and a fragment of a coding region from Jembrana Disease Virus cloned into a pEX-A128 vector (Eurofins, Ebersberg, Germany) as previously described (Yang et al. 2013) was added to all faecal DNA samples to detect any PCR inhibitors in the extracted DNA.

Statistical analysis

All analyses were performed in R 4.3.2 (R Core Team 2023). Generalised linear mixed-effects models (GLMMs) with binomial distribution and logit link were employed to test the effects of study area (categorical; four levels) and age/sex class (categorical; four levels) on the probability of infection by *G. intestinalis* or *E. bieneusi* (both binary response variables; present or absent). To account for multiple sampling within each study group, we included a random intercept for study group identity

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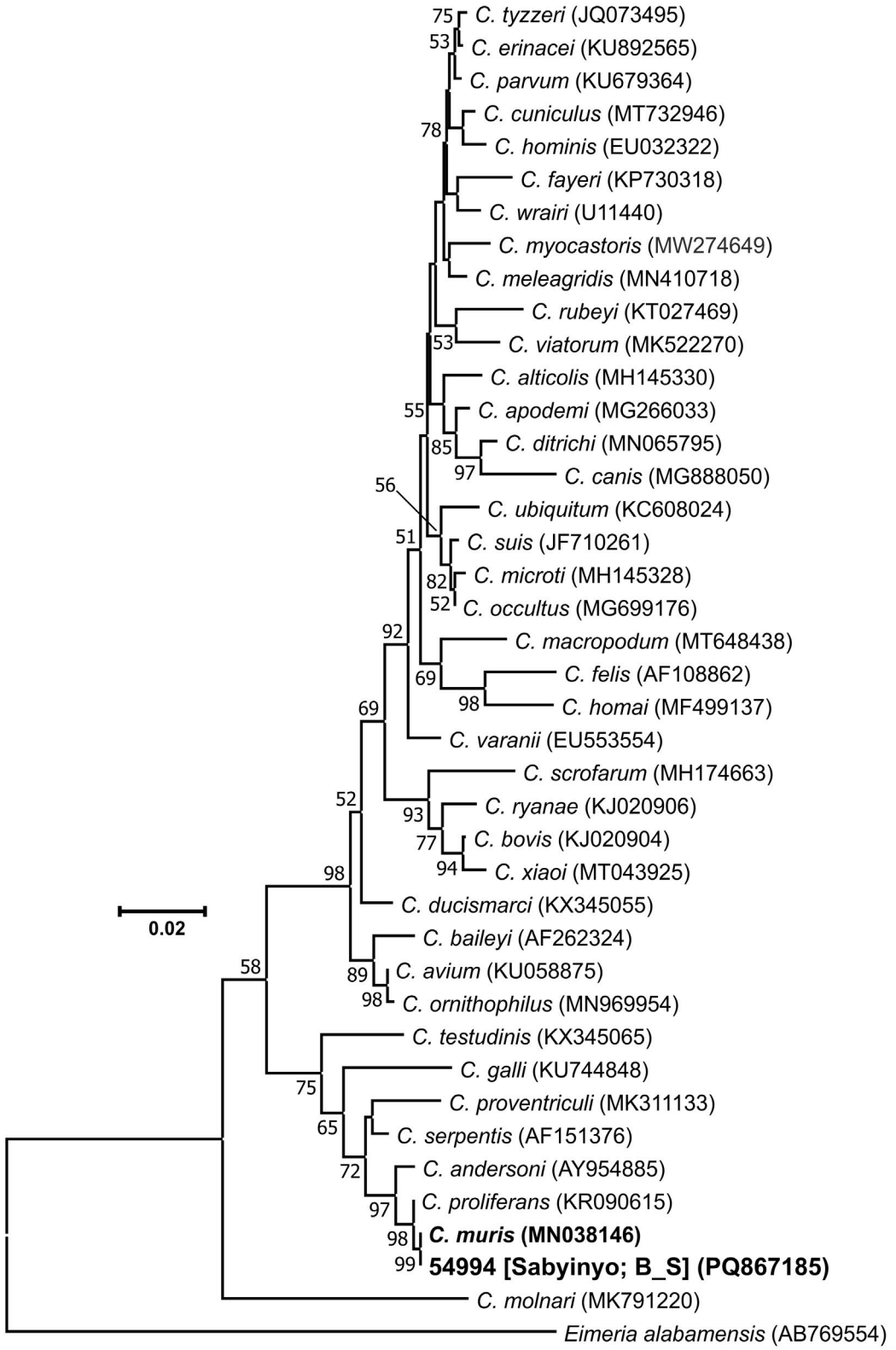


Fig. 2. Phylogenetic relationships between *Cryptosporidium muris* isolate 54994 detected in the present study (highlighted) and other *Cryptosporidium* spp. deposited in GenBank as inferred by Maximum Likelihood (ML) analyses (General Time Reversible model with a gamma distribution) of the small subunit of rDNA. Percentage support (> 50%) from 1,000 pseudoreplicates from ML is indicated next to the supported node. The tree was drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The tree was rooted with the sequence of *Eimeria alabamensis*. The GenBank Accession number is in parentheses, and the name of the gorilla group and the area of sampling location are in brackets.

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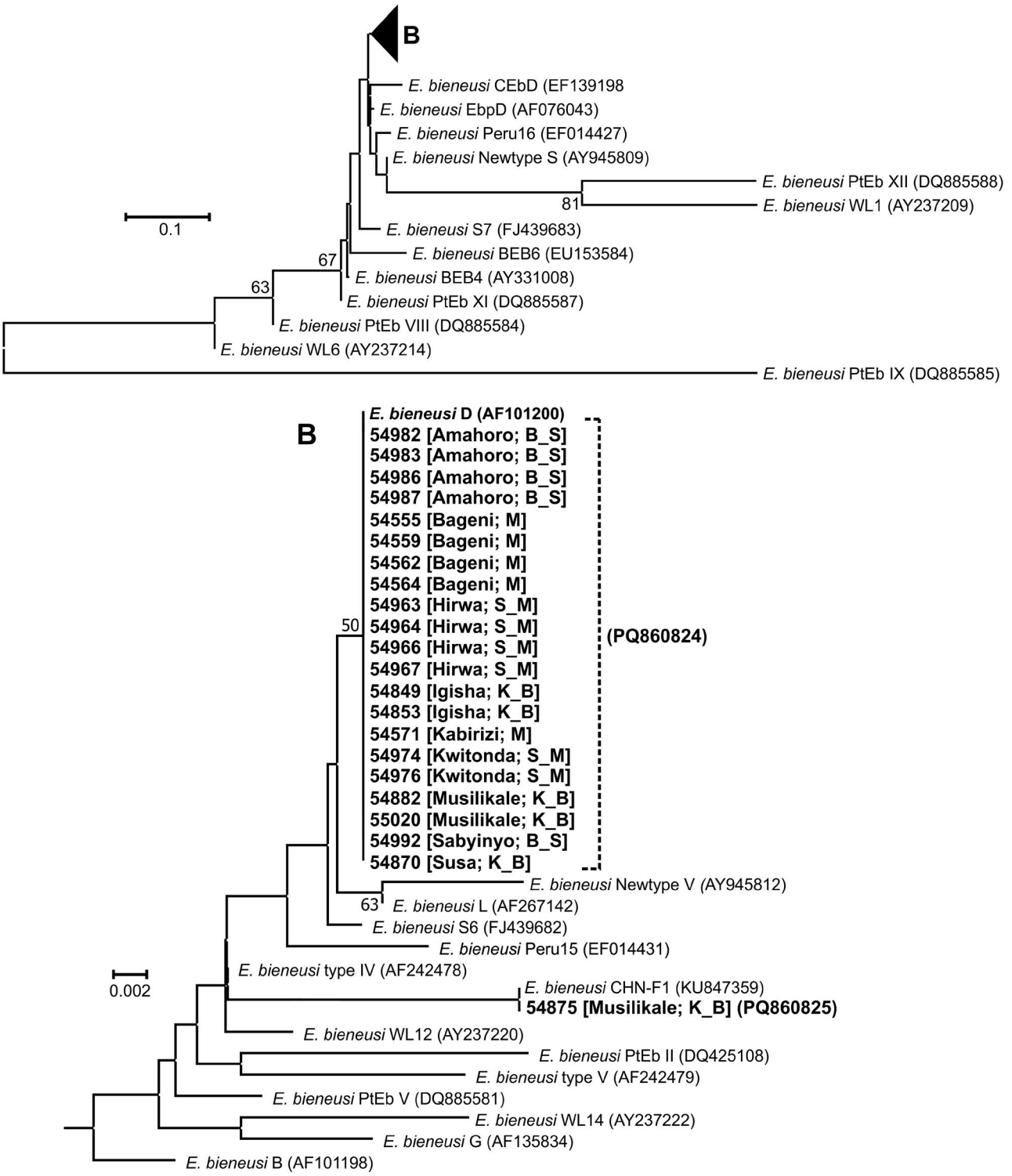


Fig. 3. Phylogenetic relationships among genotypes of *Enterocytozoon bieneusi* detected in the present study (highlighted) and other isolates deposited in GenBank as inferred by Maximum Likelihood (ML) analyses (General Time Reversible model with a gamma distribution) of the internal transcribed spacer 1. Percentage support (> 50%) from 1,000 pseudoreplicates from ML is indicated next to the supported node. The tree was drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The tree was rooted with the ITS sequence *E. bieneusi* PtEbIX genotype. Sequences obtained in this study are identified by isolate number (e.g. 54987). The GenBank Accession number is in parentheses, and the name of the gorilla group and the area of sampling location are in square brackets.

in the models. We assessed potential multicollinearity using the variance inflation factor (VIF), which was satisfactory for all predictors (VIF < 1.1). Model diagnostics showed no overdispersion (dispersion ratio = 0.8) or violations of model assumptions based on simulated residuals (Hartig 2022). Statistical

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significance was assessed using Wald Chi-square tests, and *post-hoc* pairwise comparisons of estimated marginal means were performed using the *emmeans* package (Lenth 2024).

Results

In 152 faecal samples, *Cryptosporidium*-specific DNA was detected only in one sample by nested PCR targeting the 18S rDNA gene. From this positive sample (< 1%), partial sequences of 18S rDNA confirmed the presence of *C. muris* (Table S1, Fig. 2). Quantitative PCR revealed a *C. muris* load of 2.3×10^2 OPG (Table S1). Neither PCR nor qPCR detected *Encephalitozoon*-specific DNA in any of the tested samples, but *E. bieneusi*-specific DNA was confirmed in 22 samples (14%). qPCR revealed an *E. bieneusi* load ranging from 9.1×10^1 to 1.4×10^3 SPG (Table S1). Phylogenetic trees constructed from complete ITS sequences demonstrated two distinct genotypes: 21 sequences clustered to the previously known genotype D (GenBank accession number AF101200; Fig. 3), and one sample was identical with *E. bieneusi* genotype CHN-F1 (Fig. 3). No samples tested positive for *G. intestinalis* using nested PCR, but qPCR detected *G. intestinalis* in 19 samples (13%) with low faecal protist loads ranging from 1.3×10^1 to 2.4×10^2 CPG (Table S1). Despite multiple attempts, we failed to genotype *G. intestinalis* in the positive samples.

When examining the effects of area and age/sex class on the probability of infection using GLMM, the full model for *E. bieneusi* did not perform significantly better than the null model containing only a random effect (likelihood ratio test: $\chi^2 = 7.11$, df = 6, $P = 0.31$). *Enterocytozoon bieneusi* infection probabilities did not differ among study areas (GLMM; Chi-square = 2.67, df = 3, $P = 0.44$) or age/sex classes (Chi-square = 4.07, df = 3, $P = 0.25$). Pairwise comparisons of infection probabilities (predicted marginal mean (95% CI)) in areas K_B (8% (3-20%)), B_S (20% (7-45%)), S_M (15% (6-34%)) and M (9% (3-25%)) showed no statistical differences (all $P > 0.46$). Similarly, infection probabilities in adults (20% (11-34%)), silverbacks (8% (2-28%)), infants (6% (1-24%)) and juveniles (19% (7-43%)) did not differ significantly (all $P > 0.35$).

The full model for *G. intestinalis* did not perform significantly better than the null model containing only a random effect (likelihood ratio test: $\chi^2 = 6.97$, df = 6, $P = 0.32$). *Giardia intestinalis* infection probabilities did not differ among study areas (GLMM; Chi-square = 3.20, df = 3, $P = 0.36$) or age/sex classes (Chi-square = 3.67, df = 3, $P = 0.30$). Pairwise comparisons of infection probabilities (predicted marginal mean (95% CI)) in areas K_B (6% (2-18%)), B_S (13% (4-38%)), S_M (9% (2-27%)) and M (18% (8-38%)) showed no statistical differences (all $P > 0.31$). Similarly, infection probabilities in age/sex classes of adults (12% (5-25%)), silverbacks (24% (10-45%)), infants (7% (2-25%)) and juveniles (7% (1-26%)) did not differ significantly (all $P > 0.38$).

Discussion

Using molecular diagnostics applied to faecal specimens from multiple mountain gorilla groups inhabiting the Virunga Massif, we detected *Cryptosporidium* in less than 1% of examined samples, microsporidia *E. bieneusi* in 14% of examined samples, and *G. intestinalis* in 13% of examined samples. Most studies on great ape parasites utilising molecular tools report low prevalence of *Cryptosporidium* infections in mountain gorillas and other great apes (Sak et al. 2013, Butel et al. 2015, Parsons et al. 2015). Phylogenetic analyses revealed the presence of specific DNA of *C. muris*, which primarily infects the glandular part of the stomach in rodents (mice and rats), but has also been detected in various domestic animals and mammals, including Sumatran and Bornean orangutans (*Pongo abelii* and *P. pygmaeus*) and humans (Iseki 1986, Mynářová et al. 2016, Kváč et al. 2017). However, the occurrence of this *Cryptosporidium* species in apes is not common. The detection of a single case of *C. muris* infection in mountain gorillas aligns with previous findings by Sak et al. (2014) in the study population. The presence of *C. muris* in the gorilla host could be attributed to contact with rodent faeces; however, it is difficult to distinguish between the mechanical passage of ingested oocysts and an ongoing infection (Xiao et al. 2006, Kváč et al. 2012).

The analyses of the ITS sequences revealed two genotypes of *E. bieneusi*, the more common genotype D present in several studied gorilla groups and the genotype CHN-F1 in just one sample. Both *E. bieneusi* genotypes D and CHN-F1 belong to the so-called group 1, which includes microsporidia with zoonotic potential and of public health significance (Thellier & Breton 2008, Zhao et al. 2015). The *E. bieneusi* genotype D isolated from mountain gorillas was identical to isolates from humans, Korean water deer (*Hydropotes inermis argyropus*), raccoon dogs (*Nyctereutes procyonoides*) and rhesus macaques (*Macaca mulatta*) (Sulaiman et al. 2003b, Li et al. 2016, Amer et al. 2019). In general, genotype D can infect a wide range

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of hosts, including domestic and wild animals and humans (Sak et al. 2008, Li et al. 2011, Santín & Fayer 2011, Mori et al. 2013) and has also been detected in other great apes, namely in western lowland gorillas (Sak et al. 2013), both wild and semi-captive Sumatran orangutans (Mynářová et al. 2016), chimpanzees (*Pan troglodytes*) from zoological gardens and sanctuaries (Sak et al. 2011c) and also in mountain gorillas from the same population in a previous study (Sak et al. 2014). The detected *E. bieneusi* genotype CHN-F1 was identical to isolates from raccoon dogs and blue foxes (*Alopex lagopus*) (Zhao et al. 2015, Xue et al. 2016). Its presence in mountain gorillas is surprising, marking the first recorded instance in a non-carnivore host. The potential role of humans, domestic animals, and wild animals as sources of *E. bieneusi* genotype D and *C. muris* or sympatric carnivores as sources of genotype CHN-F1 in Virunga gorillas remains speculative. This warrants further research on protist infections in sympatric wild and domestic animals, as well as humans, in the Virunga Massif and its surroundings.

The observed prevalence of opportunistic protists in Virunga Mountain gorillas was comparable to previous studies of great apes using molecular methods (Sak et al. 2013, Mynářová et al. 2016). However, the prevalence of microsporidia and *Cryptosporidium* infections is only partially consistent with the previous study of Sak et al. (2014) in the same gorilla population, even if comparing only the results from the Rwandan part of Virunga, which was surveyed by Sak et al. (2014). They detected multiple genotypes of *E. bieneusi* (18%), namely EbpA, C, D, gorilla 2, 4-8, and *E. cuniculi* genotypes I and II (11%), *C. muris* and *C. meleagridis* (less than 1%) in samples of studied gorillas collected in 2007, while we found no *Encephalitozoon* infections, only two genotypes of *E. bieneusi* and one *Cryptosporidium* species in samples collected in 2018. In our study, the prevalence of *E. bieneusi* in the gorilla groups in the Rwandan part of Virunga was only 15%. Because we did not find any *Encephalitozoon* infections, the overall prevalence of microsporidia in this area is also 15%, while Sak et al. (2014) reported 29% prevalence of microsporidia. Thus, in contrast to our prediction, we demonstrate a decline in prevalence and diversity, especially of microsporidia infections, in the Virunga gorilla population between 2007 and 2018 (Sak et al. 2014).

We speculate that *E. bieneusi* genotype D might have gradually replaced other genotypes found previously and become dominant in mountain gorillas across the Virunga Massif. A similar situation was observed in captive apes, where genotypes common in the wild were replaced by those from other hosts that were in contact with captive apes (Sak et al. 2011a). The failure to detect *Encephalitozoon* infections in the study was unexpected. Several studies have commonly revealed *E. cuniculi* infections in great apes, including the Virunga gorilla population (Sak et al. 2013, 2014, Mynářová et al. 2016), leading to the assumption that *Encephalitozoon* infections might be present in all ape populations (Cibot et al. 2021). *Encephalitozoon cuniculi* genotypes are not host-specific and have been identified in a range of animals, including rodents, lagomorphs, carnivores, humans, non-human primates, and birds (Canning & Hollister 1987, Reetz 1993, Kašičková et al. 2009). The reasons for failing to detect *Encephalitozoon* infections in this study remain unclear, but PCR inhibition was ruled out since IAC and other parasites were detected.

In comparison to 2007 (Sak et al. 2014), we found an increase in *G. intestinalis* infections, but this could have been due to the implementation of a more sensitive method, specifically qPCR (Capewell et al. 2020, Belkessa et al. 2021). The faecal protist load was low (10^1-10^2), and we demonstrated that qPCR is more effective than nested PCR for detecting low-burden *G. intestinalis* infections in wild hosts. This finding is consistent with our previous study, which has shown qPCR's higher sensitivity compared to conventional PCR, but this approach does not allow for the identification of assemblages (Brožová et al. 2023). Due to different diagnostic methods in previous studies, we did not attempt to compare the *Giardia* prevalence with other studies that did not employ qPCR. Future research efforts should aim to develop sensitive methods to not only detect *G. intestinalis* but also identify specific assemblages.

There is a lack of similar studies focusing on long-term changes in studied protist infections in wild hosts, except for Sak et al. (2013), which found no changes in microsporidia infections in western lowland gorillas over three years. Microsporidia are ubiquitous, and many gorillas become infected during their lifetime, though the quantity and frequency of spores shed by immunocompetent individuals are low, and spores are shed intermittently; thus, repeated sampling is desirable (Sak et al. 2011a, b). To gain insight into the epidemiology of the studied protists across the whole Virunga Massif, we sampled faeces from the nests of unidentified individuals at night nest sites like Sak et al. (2014). However, we would recommend repeated sampling of known individuals for future long-term monitoring to provide more details about

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microsporidia, but also *Cryptosporidium* and *Giardia* infections in endangered mountain gorillas.

Contrary to our prediction, we report no difference among study areas across the Virunga Massif in the occurrence of microsporidia and *Giardia*. We thus conclude that the described changes in host population dynamics (Caillaud et al. 2014, 2020) had no impact on the epidemiology of studied protist infections, unlike strongylid nematodes (Mason et al. 2025). Microsporidia and *Giardia* infections also probably do not play a role in the increase of observed gastrointestinal problems reported in Virunga gorillas. For now, increased absolute abundance of pathogenic strongylid nematodes seems to be the main cause of observed health problems (Petrželková et al. 2021, 2022, Mason et al. 2025).

We also did not observe any differences in the occurrence of focal protists among defined sex/age categories. The literature is inconclusive on the sex-dependent prevalence of the studied protists in human and animal populations. Human populations exhibited a higher parasite load with increasing age, likely due to cumulative exposure and a limited immune response (Sak et al. 2011a, 2017). In studies on different mouse populations, the findings were also inconclusive; in some populations, the effect of sex was evident, while in others, it was not (Sak et al. 2011b). Previous studies have also shown inconsistencies regarding the impact of age on *Giardia* infections. Some studies reported no differences across age categories (e.g. horses: Santín et al. 2013) while others found higher prevalence in subadults compared to adults (e.g. livestock: Johnston et al. 2010). In humans, some studies reported higher prevalence in adults (Johnston et al. 2010, Ferguson et al. 2020, Brožová 2021) while others found higher prevalence in children compared to other age groups (Oyerinde et al. 1977). Among non-human primates, no age-related differences in *Giardia* infections were found in black and gold howler monkeys (*Alouatta caraya*) living in fragmented forests (Alegre et al. 2021). We were unable to analyse the impact on *Cryptosporidium* infections due to the low prevalence observed.

Parasite infections in wild non-human primate populations rarely cause host mortality and are often asymptomatic. However, the cases of gastrointestinal diseases and even mortalities in mountain gorillas associated with strongylid nematodes (Muhangi et al. 2021, Petrželková et al. 2021) underscore the importance of regular pathogen monitoring to inform and support effective conservation strategies. These plans should include regular monitoring of parasite infections and consider the spread of parasites among gorillas through environmental contamination to ensure sustainable protection and the survival of the endangered Virunga Mountain gorillas. Although we conclude that *Cryptosporidium*, microsporidia and *Giardia* infections do not currently pose a threat to the Virunga Mountain gorilla population, we suggest continuing the monitoring of these protist infections with the implementation of repeated individual sampling. We also suggest identifying the sources of infections via systematic pathogen monitoring of sympatric wildlife, as well as domestic animals and humans living in proximity to the park.

Acknowledgements

We are thankful to the Rwanda Development Board, the Uganda Wildlife Authority, the Uganda National Council of Science and Technology, and the Institut Congolais pour la Conservation de la Nature for their long-term support and for their permissions to conduct this research. We are indebted to the field staff of the Dian Fossey Gorilla Fund, Gorilla Doctors, Rwanda Development Board, Virunga National Park, Bwindi Impenetrable National Park and Mgahinga Gorilla National Park for their tireless support in collecting faecal samples. The study was supported by the Czech Science Foundation (22-16475S, 21-23773S) and the U.S. Fish and Wildlife Service Division of International Affairs, Grant no. F17AP00964. Carine Uwamahoro was supported by a Wildlife Conservation Network Veterinary Scholarship. Klára Petrželková and Barbora Pafčo were also supported by institutional support from the Institute of Vertebrate Biology, Czech Academy of Sciences (RVO:68081766).

Author Contributions

Carine Uwamahoro: data curation, formal analysis, investigation, resources, visualisation, writing – original draft, writing – review & editing; Nikola Holubová: investigation, methodology; Milan Jirků: investigation, methodology, writing – review & editing; Bohumil Sak: methodology, writing – review & editing; Dana Květoňová: investigation, methodology; Peter Samaš: data curation, formal analysis, methodology, writing – review & editing; Barbora Červená: investigation, writing – review & editing; Dušan Romportl: visualisation, writing – review & editing; Winnie Eckardt: investigation, resources, writing – review & editing; Felix Ndagijimana: investigation, resources; Richard Muvunyi: investigation, resources; Prosper Uwingeli: investigation, resources; Jeff Mapilanga: investigation, resources; Arthur Kalonji: investigation,

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resources; Robert Aruho: investigation, resources; Michael Cranfield: conceptualisation, investigation, resources; Kirsten Gilardi: funding acquisition, resources, writing – review & editing; Julius Nziza: investigation, resources; Benard Ssebide: investigation, resources; Eddy Kambale Syaluha: investigation, resources; Barbora Pafčo: investigation, writing – review & editing; Kateřina Jirků: conceptualisation, methodology, supervision, visualisation, writing – original draft, writing – review & editing; Martin Kváč: conceptualisation, funding acquisition, methodology, supervision, visualisation, writing – original draft, writing – review & editing; Klára J. Petrželková: conceptualisation, funding acquisition, methodology, supervision, visualisation, writing – original draft, writing – review & editing.

Data Availability Statement

All sequence data generated during this study have been deposited in the GenBank database. The 18S rDNA gene sequence is available under the accession number PQ867185. Internal Transcribed Spacer (ITS) region sequences are available under the accession numbers PQ860824 and PQ860825. These data are publicly accessible and can be retrieved from the NCBI GenBank repository.

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Supplementary online material

Table S1. The occurrence, genotypes and faecal protist load of *Cryptosporidium* spp., *Enterocytozoon bieneusi*, *Encephalitozoon* spp. and *Giardia intestinalis* in faecal samples of Virunga Mountain gorillas were detected by nested PCR and qPCR. Only positive samples are listed. Karisimbi-Bisoke (K_B), Bisoke-Sabyinyo (B_S), Sabyinyo-Muhavura (S_M), Mikeno (M). *species/genotype/assemblage; **Ct (cycle threshold) value/protist load expressed as number of *Cryptosporidium* oocysts (OPG), *Giardia intestinalis* cysts (CPG) and microsporidia spores (SPG) per gram of faeces. '-' represents a negative result of PCR/qPCR (https://www.ivb.cz/wp-content/uploads/JVB-74-2025-Uwamahoro-C.-et-al.-Table-S1.pdf).

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Pafčo, B., Kreisinger, J., Romportl, D., Mason, B., Červená, B., Uwamahoro, C., Eckardt, W., Ndagijimana, F., Nziza, J., Kambale Syaluha, E., Ssebide, B., Noheri, J.B., Bahizi, M., Okello, R.O., Nizeyimana, F., Lukusa, J.-P., Gilardi, K., Muvunyi, R., Uwingeli, P., Mudakikwa, A., Mapilanga, J., Kalonji, A., Aruho, R., Hickey, J., Šlapeta, J., Modrý, D., Lowenstine, L., Hasegawa, H., Cranfield, M., Petrželková, K.J.

2026

Emerging pathogenic strongylid infections in Virunga Massif mountain gorillas: role of environmental stressors.

J. Appl. Ecol. 63, e70258. https://doi.org/10.1111/1365-2664.70258

Check for updates

Received: 31 March 2025 | Accepted: 13 November 2025

DOI: 10.1111/1365-2664.70258

Journal of Applied Ecology

RESEARCH ARTICLE

Emerging pathogenic strongylid infections in Virunga Massif mountain gorillas: Role of environmental stressors

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Funding information
The Czech Science Foundation, Grant/Award Number: 18-24345S and 22-16475S; U.S. Fish and Wildlife Service, Grant/Award Number: F17AP00964; Institute of Vertebrate Biology, Czech Academy of Sciences, Grant/Award Number: RVO:68081766; Masaryk University, Grant/Award Number: MUNI/A/1602/2023

Handling Editor: Bret Elderd

Abstract

1. Conservation success stories, such as the recovery of the isolated populations of mountain gorilla (*Gorilla beringei beringei*), are increasingly threatened by emerging health challenges. In the Virunga Massif, strongylid-associated gastrointestinal disease has been linked to significant morbidity and mortality of mountain gorillas, posing a critical threat to this population's long-term survival.
2. Using metabarcoding of faecal samples (~65% of the population) and spatial and habitat analyses, we examined the composition and drivers of strongylid nematode communities and their role in clinical disease emergence.
3. We identified *Hyostrongylus* nematodes, known pathogens in suids, as the primary contributors to gastric lesions observed in necropsied gorillas. Spatial variation in strongylid communities is correlated with environmental gradients, indicating that environmental factors such as climate and vegetation play a significant role in shaping strongylid community composition. Additionally, limited genetic diversity resulting from historical population bottlenecks may negatively impact the health of gorillas affected by parasites.
4. Our findings suggest that the high absolute abundance of pathogenic *Hyostrongylus* is concentrated in specific high-altitude regions, aligning with observed cases of severe gastritis.

Barbora Pafco and Jakub Kreisinger contributed equally to this work.

†Deceased.

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5. *Synthesis and applications*. These findings highlight an emerging health threat for mountain gorillas, with implications for other isolated wildlife populations facing similar ecological and evolutionary pressures. The study calls for continued monitoring of parasitic diseases in wildlife populations, especially those in fragmented habitats with limited capacity for expansion, to mitigate potential risks to their conservation.

KEYWORDS
gastrointestinal, helminth, metabarcoding, nematode, parasite, primate, wildlife disease

1 | INTRODUCTION

One of the greatest challenges for conservationists in this century is the protection of biodiversity and the remaining natural habitats (Maxwell et al., 2020). With habitat destruction, fragmentation and even loss, an increasing proportion of wildlife survives only in fragments of their natural environment (Gonçalves-Souza et al., 2020; Sloan et al., 2014). While extreme habitat changes threaten species with extinction, conservation efforts can promote survival and even recovery of some populations (Bejder et al., 2016; Hays, 2004). However, remaining populations, especially isolated ones, face the constant threat of habitat scarcity, which can lead to limited food sources, increased stress due to home range overlaps, and overall changes in animal behaviour, generating novel processes to regulate population growth, such as infectious diseases (Calizza et al., 2017; Herrera & Nunn, 2019). One example of a great conservation success is the recovery of the mountain gorilla (*Gorilla beringei beringei*), that is considered a flagship species for wildlife conservation (Robbins et al., 2011). One mountain gorilla population is found in the Virunga Massif at the borders of Rwanda, Uganda and the Democratic Republic of the Congo (DRC), and the other in the Bwindi–Sarambwe ecosystem in Uganda and DRC. Due to intensive conservation efforts, including veterinary interventions, mountain gorillas are the only great ape species in the wild whose numbers are gradually increasing, with the trend being most clearly documented for the Virunga population (Granjon et al., 2020). This positive trend, observed since the late 1980s, led the IUCN in 2018 to reclassify their conservation status from critically endangered to endangered (Plumptre et al., 2019).

Despite this success in gorilla conservation, major threats persist. Both legal and illegal human activities in situ as well as climate change can negatively affect gorillas and their habitats (Belfiore et al., 2015; Plumptre et al., 2019; van Gils & Kayijamahe, 2010), which can lead to the introduction of new diseases or changes in the epidemiology of already harboured pathogens, with potential health consequences (Petrželková et al., 2022). Moreover, the inability of gorillas to expand their geographical range beyond park boundaries as their numbers grow can alter social dynamics with potentially negative outcomes (Caillaud et al., 2020; Morrison et al., 2022). Transmission of human diseases (particularly respiratory diseases) (Grützmacher et al., 2016; Köndgen et al., 2008; Scully et al., 2018) and the risk of an Ebola virus outbreak have been considered the major disease threats for mountain gorillas (Zimmerman et al., 2023). However, in recent years, we have observed what may be an increasing occurrence of parasite-associated gastrointestinal diseases in both mountain gorilla populations, ranging from moderate or subclinical to fatal cases (Muhangi et al., 2021). In Virunga Massif, up to 25 cases of fatal gastritis were recorded during the most intense 8-year period of gastritis (2008–2016), based on histopathological examinations of necropsied gorillas. These gorilla fatalities are alarming, with only 604 individuals the estimated number of gorillas in Virunga Massif (Granjon et al., 2020). The majority of severe gastritis cases (84%) occurred within a single region, among groups ranging between Mt. Karisimbi and Mt. Bisoke in *Hagenia* forest and *Hypericum* woodland. The assumption was that gastric trichostrongylid nematodes were involved in gorilla gastritis through the formation of gastric lesions (including neoplasia), which occurs in a number of domestic and wild mammals (including primates) (Collett et al., 2000; Dennis et al., 2006; Smetana & Orihel, 1969). The lesions with trichostrongylids were associated with chronic gastric mucosal proliferations that caused debilitation and contributed to mortality of mountain gorillas. The trichostrongyid nematodes were observed in the majority of gastritis cases, while gastric trichostrongyles were not found histologically in cases without gastritis (Muhangi et al., 2021) (Lowenstine, unpublished data). In contrast, in the strongylid communities of free-ranging western lowland gorillas trichostrongylids are very rare (Mason et al., 2022), and to our knowledge, neither clinical symptoms nor fatal cases of strongylid nematodes have been observed in any population of free-ranging western lowland gorillas. This absence is notable given that strongylid communities have been surveyed across multiple western lowland gorilla populations and sites in Central Africa (Mason et al., 2022).

Mountain gorillas occur in only two small isolated populations, inhabiting two protected forest islands surrounded by agricultural landscapes and dense human populations (Hickey et al., 2020). Virunga gorillas live at high densities, experiencing cooler temperatures, high rainfall and frequent inter-group encounters (Caillaud et al., 2020). In contrast, western lowland gorillas occupy a vast range of lowland tropical and swamp forests across Central Africa, typically at lower densities, with more dispersed social groups (Doran & McNeilage, 1998; Robbins & Robbins, 2018). Despite this ecological and geographical diversity, western lowland gorillas show

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remarkably consistent strongylid community composition across populations (Mason et al., 2022), where they are consistently dominated by *Oesophagostomum* and *Necator* species with no clinical manifestations. On the other hand, the diversity of strongylid nematodes in mountain gorillas has been rather poorly studied, with the majority of prior research lacking species or even genus-level identification (Ashford et al., 2016; Kalema-Zikusoka et al., 2005; Rothman et al., 2008; Sleeman et al., 2000).

To understand gastrointestinal health problems and explain clinical case emergence among mountain gorillas inhabiting the Virunga Massif, we investigated the determinants of their strongylid communities and identified nematodes involved in lesions from histological samples of necropsied gorillas. We described the complex strongylid communities using metabarcoding via high-throughput sequencing (HTS) (Mason et al., 2022; Petrželková et al., 2021) and assessed the absolute abundance of strongylid genera by combining the relative abundance of genera with strongylid egg counts. First, we hypothesized that the occurrence of gastrointestinal disease in mountain gorillas is due to different strongylid communities compared to western lowland gorillas (Mason et al., 2022) which do not show any symptoms associated with these parasites. Second, we hypothesized that strongylid communities in the Virunga Massif are dominated by gastric strongylid taxa that can cause gastritis in infected animals (Petrželková et al., 2021). Third, based on previous studies using simple coproscopical methods that provided insight into strongylid epidemiology in mountain gorillas (Petrželková et al., 2021, 2022), we hypothesized that group identity, and the factors which vary within (sex/age class and season) and between groups (group size, spatial configurations and environmental gradients, vegetation and host densities within home range), have a significant impact on strongylid communities and individual strongylid genera in gorillas across the Virunga Massif, consequently affecting clinical case emergence in particular areas.

2 | MATERIALS AND METHODS

2.1 | Study site, sample and data collection

The study was carried out in the Virunga Massif (451 km^2), which spans three contiguous national parks: Volcanoes National Park (VNP) in northern Rwanda, Virunga National Park (ViNP) in eastern Democratic Republic of the Congo (DRC) and Mgahinga Gorilla National Park (MGNP) in southwestern Uganda. In Virunga Massif, the altitude range is 1885 – 4091 m above sea level, with vegetation types mainly determined by elevation (Owiunji et al., 2005), average temperatures are from 6 to 17°C and precipitation from 1499 to 2186 mm/year (Fick & Hijmans, 2017). Virunga Massif has a complex mosaic of vegetation types: the most abundant vegetation type in ViNP is mixed forest, whereas in VNP it is bamboo, followed by *Hagenia* forest and *Hypericum* woodland, while MGNP is dominated by mixed forest and bamboo (Owiunji et al., 2005). There are two rainy seasons (approximately March–May and September–November) and two dry seasons (approximately December–February and June–August). Sixteen of the twenty-nine studied gorilla groups within the Virunga Massif regularly use the highest altitudes – between 2800 to 3450 m above sea level (a.s.l.), especially on the Rwandan side of the mountain range, where temperatures can drop to 0°C (for additional information on the study site see Petrželková et al., 2021, 2022).

In 2018, we collected fresh faecal samples from habituated mountain gorilla groups (identified by name): twenty groups from VNP, eight groups from ViNP and one group from MGNP (Figure 1, Table S1). To assess the potential influence of season on strongylid communities, gorilla groups in ViNP and VNP were sampled twice, once in the dry season (January/February) and once in the wet season (September/October) (Table S1). The gorilla group from MGNP was sampled only once, in the wet season. Faecal samples were collected in the morning from gorilla nests made the previous night, placed in plastic bags, and labelled with the estimated sex/age class (silverback >12 years, adult >8 years, subadult/juvenile >3.5–8 years, infant 0–3.5 years) based on the size of faecal lobes (Gray et al., 2013; Sinayitutse et al., 2021). On the same day of collection, faecal samples were transported to field laboratories in Musanze (Rwanda), Goma (DRC) or Kisoro (Uganda), where aliquots were fixed in 96% ethanol and stored in a refrigerator immediately after arrival. The strongylid eggs were quantified within a maximum of 48 h post estimated defecation, using a Mini-FLOTAC© device and eggs per gram of faeces (EPG) were calculated (Cringoli et al., 2017; Petrželková et al., 2021). The number of EPG can be used as a proxy for nematode infection intensities/abundance in both domestic and wild animals, including non-human primates (Burgunder et al., 2017; Chapman et al., 2006). Data on gorilla group size (number of individuals) were obtained from Dian Fossey Gorilla Fund, Gorilla Doctors, Rwanda Development Board, Uganda Wildlife Authority and Institut Congolais pour la Conservation de la Nature databases. Aliquots were later shipped to the University of Veterinary Sciences Brno, Czech Republic for further molecular analyses, where they were stored at −20°C prior to DNA isolation.

2.2 | Ethics statement

The research complied with the legal requirements of Rwanda, Uganda and Democratic Republic of the Congo. This research involved only non-invasive work with wild non-human primates. All work was approved and conducted in collaboration with the Rwanda Development Board (0010/RDB-T&C/V.U/18, 2.00/October/RDB-T&C/V.U/18), the Uganda Wildlife Authority (COD/96/05), the Uganda National Council of Science and Technology (NS644), and the Institut Congolais pour la Conservation de la Nature (1193/ICCN/DG-INT/JMT/03/020/2018), and undertaken in accordance with their respective rules and regulations. Field site access was facilitated by in-country collaborators with access permissions and

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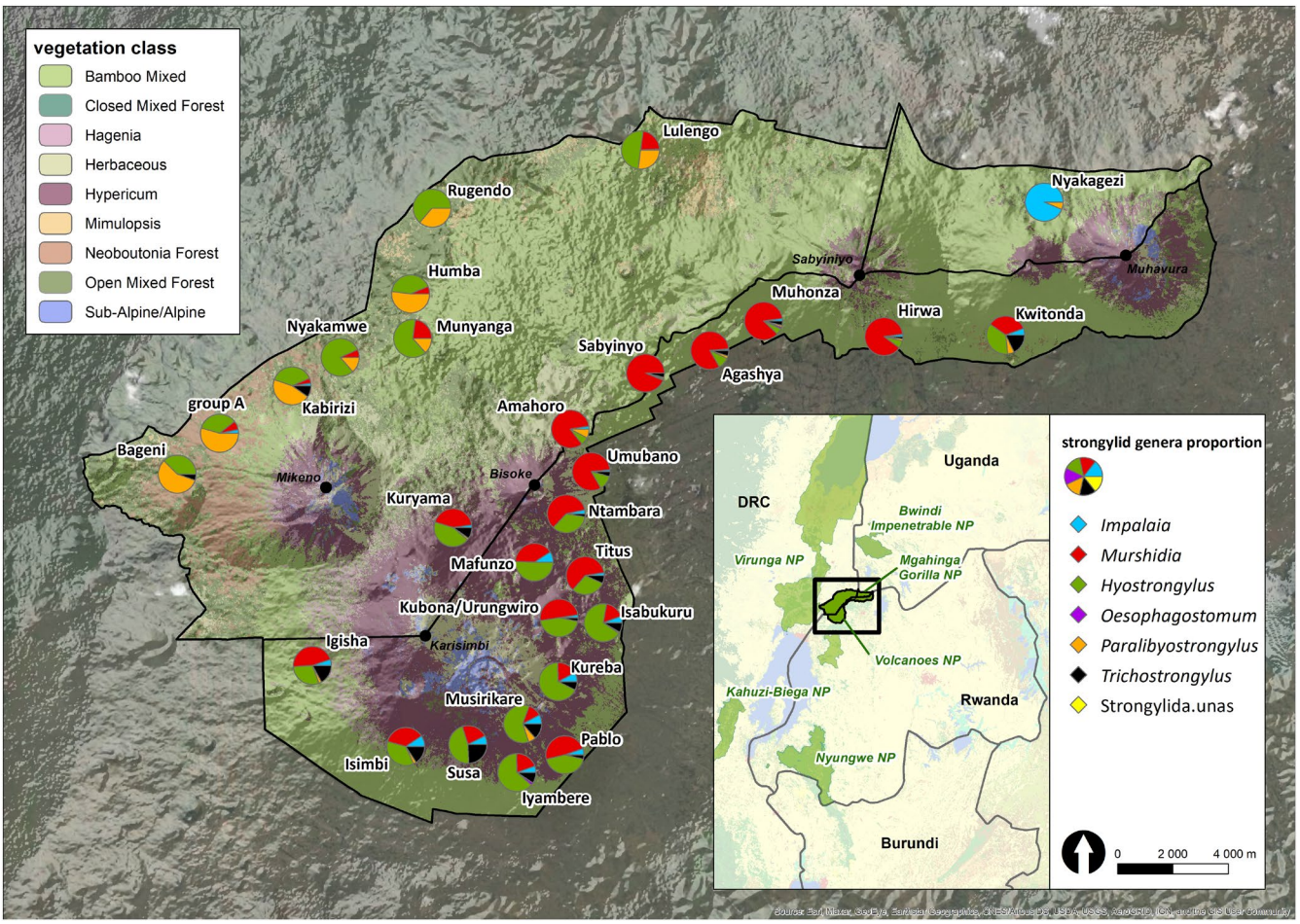


FIGURE 1 Proportion of strongylid genera in gorilla groups across Virunga Massif. Location of groups is expressed as centroids of their 50% kernel home ranges and volcanos are shown in black. Vegetation data were adopted according to WWF-Germany and IGCP (WWF-Germany & IGCP, 2017); boundaries of protected areas were derived from ProtectedPlanet.net database. Map was created using ArcGIS Desktop 10.8 (ESRI 2020. ArcGIS Desktop: Release 10.8. Redlands, CA: Environmental Systems Research Institute; esri.com).

long-term research activities in each locality, all of whom are included as co-authors.

2.3 | Histopathological examination

Lesions associated with gastritis, collected during necropsies, were cut open and examined under the microscope together with the nematodes found, for more details see Muhangi et al. (2021).

2.4 | Spatial and habitat analyses

Geographic location (GPS) data for each gorilla group were collected using IMPACT 2.0 (Medical Decision Logic, Inc.), a syndromic health care monitoring system developed by Gorilla Doctors for collecting daily health data on individual animals and during daily Ranger-Based-Monitoring established in the three countries. For this analysis, we use the GPS data point recorded at arrival to each group during daily monitoring, which is usually close to the night nest, by Rwanda Development Board, Dian Fossey Gorilla Fund, Gorilla Doctors and Institut Congolais pour la Conservation de la Nature staff. Daily monitoring of the study groups also provided information on the number of individuals within the groups, which allowed us to estimate the local host and group densities.

As the data provided were heterogeneous in terms of format and other attributes, these were first standardized and transformed into a spatially explicit database in ArcGIS™. First, 50% and 95% kernel annual home ranges for each gorilla group were derived using Home Range Tools for ArcGIS using smoothing factor 200 m as default. The calculation of annual home ranges included GPS data points from a 12-month period before the sampling period. A set of explanatory extrinsic variables was prepared and calculated per kernel home range of each gorilla group, using zonal statistics for four groups of factors. The abiotic factors (1) captured basic environmental gradients, elevation, mean annual temperature and mean annual precipitation (source: WorldClim 2.0 verified by CRU TS 4.04 database). The vegetation (2) was categorized into several types: pure bamboo, mixed bamboo, *Hagenia* forest, *Hypericum* woodland, herbaceous, meadow, *Mimulopsis*,

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closed mixed forest, open mixed forest, *Neobutonia* forest, subalpine and alpine, based on data collected during the Virunga Massif 2015–2016 survey (Hickey et al., 2018). Geo-referenced locations of vegetation types collected during this survey were used as ground-truth data in a supervised landcover classification of Sentinel 2 satellite imagery (https://dataspace.copernicus.eu/data-collections/copernicus-sentinel-data/sentinel-2) (Figure 1) (WWF-Germany & IGCP, 2017). We calculated the percentage of the vegetation types for the kernel annual home range of each gorilla group. The spatial configuration (3) included home range size and geographical coordinates of the centroid of the home range; as home ranges often overlap, exclusive area was calculated to express the home range that was not shared with other gorilla groups. Last, population densities (4) with kernel densities of both individuals as well as gorilla groups were calculated using spatial analysis for the whole project area; average densities for the home range of each gorilla group were then derived using zonal statistics. We found significant correlations among 50% and 95% kernel home range sizes and exclusive home range size, as well as between gorilla individual and group densities; hence, only factors for 50% kernel home range size (herein referred to as core annual home range size) and gorilla individual densities within the core home range (herein referred to as gorilla density) were utilized in the statistical analyses.

2.5 | DNA isolation and sequencing

Total genomic DNA from faecal samples was extracted using the PowerSoil DNA isolation kit (Qiagen Company, Germany). PCR amplification was performed on the second internal transcribed spacer (ITS-2) of nuclear ribosomal DNA with forward Strongyl_ITS-2_F (acg tct ggt tca ggg ttg) and reverse primer Strongyl_ITS-2_R (atg ctt aag ttc agc ggg ta) (Pafčo et al., 2018, 2019). High-throughput sequencing (HTS) libraries were generated using a two-step-PCR approach (Pafčo et al., 2018) following the Nextera primer design. Analyses were carried out in two technical replicates with different tag primer barcodes. DNA extractions from strongylid negative human faeces and water were used as negative controls and synthetic DNA templates as positive controls (Pafčo et al., 2018). The final library was sequenced using MiSeq Reagent Kit v3 (2×300 bp pair-end reads, 600 cycles) by Illumina MiSeq platform.

2.6 | Bioinformatic analyses

We trimmed gene-specific primers using *skewer* (Jiang et al., 2014). Next, low-quality reads (expected error rate >2) were eliminated, and the filtered data set was denoised using *dada2* (Callahan et al., 2016) to obtain Amplicon Sequence Variants (ASV). Naive Bayesian *RDP classifier* (Wang et al., 2007) implemented in the *dada2* pipeline was used for taxonomic assignment of ASV with 80% posterior confidence. The reference training data set for the taxonomic assignation represented a collection of strongylid ITS-2 sequences that were either produced by our team (Supporting Information Text S1) or downloaded from the National Center for Biotechnology Information database (200 top blast hits for each of our ASV, environmental samples were excluded). To avoid inflation of strongylid diversity due to PCR/sequencing artefacts that were not corrected by *dada2*, we considered only those ASV that were consistently present in both technical duplicates for a given sample (Pafčo et al., 2018). We also excluded ASV that did not correspond to the Strongylida order. Furthermore, we removed one sample in which the number of high-quality sequences was low (<700) after all filtering steps. Read counts for ASV corresponding to the same genus were grouped within the resulting abundance matrix. Consequently, all further analyses focused on the variation in read counts among different strongylid genera. As different ASV for the same genus mostly corresponded to the same species, this step considerably reduced taxonomic redundancy of the dataset.

2.7 | Statistical analyses

Two analytical approaches were applied to characterize strongylid communities at the genus level. First, we used an abundance matrix comprising read counts for each strongylid genus and each sample as an input for statistical analyses. To account for uneven sequencing depth, we converted read counts for a given genus in each sample to their relative abundance (i.e. by dividing read count for specific genus by total read count for a given sample). As a complementary approach, we included information on strongylid infection intensities into the community abundance matrix. Specifically, we multiplied the EPG values by proportions of all genera in a given sample. Consequently, after this transformation resultant strongylid profiles reflected egg abundance of specific ASV in 1 g of faeces and hereafter it is referred to as absolute abundance.

We evaluated the effect of gorilla group identity on variation in strongylid community composition. The insight into variation of strongylid community composition was provided using Principal Coordinate Analysis (PCoA) run on Bray–Curtis dissimilarities among sample profiles. Subsequently, the variation in community composition explained by gorilla group identity was tested by PERMANOVA using a matrix of Bray–Curtis dissimilarities as a response variable. Further analyses were performed for VNP and ViNP (with exception of Group A, which was not systematically monitored to get enough GPS data) groups only. No GPS data for the only MGNP group was available at the time of the study. Thus, we could not calculate the environmental and other characteristics for those two gorilla groups and had to exclude them from further analyses. We tested the impact of predictors that varied within gorilla groups (sex/age class and season) on strongylid community composition by PERMANOVA with group identity included as constraints for permutation (i.e. 'strata').

Next, we tested predictors that differed between groups. In this case, stratified permutations were not applicable for such analyses and thus, we used Redundancy Analysis (RDA) (van den

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Wollenberg, 1977). There, we calculated the PCoA centroids of each gorilla group and used them as the response in the RDA (van den Wollenberg, 1977). The Redundancy Analysis predictors included the effects of spatial proximity, core home range size and vegetation structure. The final RDA model was constructed using a stepwise forward selection procedure (vegan::ordistep() function). In the RDA, spatial proximity was represented by geographic distances between social groups transformed by Principal Coordinates of Neighbourhood Matrix. Vegetation structure was represented by scores for the first two Principal Component Analysis (PCA) axes, which were calculated based on the proportions of vegetation types within the core home ranges of each gorilla group (PC1 and PC2; explaining 53.0% and 28.2% of the total variation, respectively). The PC1 axis was negatively correlated with *Hypericum* woodland and positively correlated with bamboo cover (Figure S1). The PC2 axis was negatively correlated with the proportion of mixed forest. PC1 and PC2 scores did not differ between dry and rainy seasons (paired t-test for PC1 and PC2 axis scores: $p > 0.5$ in both cases), suggesting that there is no statistical seasonal shift in the utilization of vegetation types. Therefore, the annual average values for each gorilla group were used. Abiotic factors were not considered as RDA predictors (e.g. average temperature, precipitation and altitude) as they were all strongly correlated with PC1 and/or PC2 (Table S3).

Finally, we analysed the effect of the selected predictors (group size, season, core home range size, scores for PC1 and PC2 for vegetation and gorilla density) on the presence, relative abundance and absolute abundance of dominating individual strongylid genera (genera that were detected in >10% of samples) by spatially explicit Bayesian Joint Species Distribution Models (JSDM) from R package *Hmsc* (Tikhonov et al., 2020), which assumed random variation among gorilla groups and samples, as well as geographic autocorrelation in strongylid community composition among gorilla groups. We applied a hurdle modelling approach, that is we first analysed variation in absence/presence of strongylid genera employing JSDM with probit link function. Next, using JSDM for log-normal Poisson distribution, we focused on variation in relative and absolute strongylid abundance for a subset of positive samples. Bayesian Joint Species Distribution Models were run on two independent Markov Chain Monte Carlo chains each consisting of 5,050,000 iterations in total. The first 50,000 iterations were discarded as burn-in and the thinning intervals were set up to 5000 steps. Chain convergence for each parameter was assessed by visual inspection of trace plots as well as by corresponding effective sample sizes and Gelman-Rubin's convergence diagnostic. Model parameters for which the 95% posterior confidence intervals did not overlap with zero were considered strongly supported.

3 | RESULTS

We identified trichostrongylid nematodes involved in gastric lesions as *Hyostrongylus* based on the morphology of sectioned body appearing in the pathological sections (Hoberg et al., 1993), specifically by presence of synlophe ridges (Figure 2).

After quality-filtering, the number of high-quality reads in the resultant relative abundance matrix was 17,515,710, with median sequencing depth being 30,213 reads per sample (range = 794–175,647). Altogether, we gathered ITS-2 profiles for 561 samples and determined that strongylid communities in the Virunga Massif gorilla population were dominated by intestinal *Murshidia* (average proportion of the reads being 38.45%) and gastric *Hyostrongylus* (36.01%), followed by gastric *Paralibyostrongylus* (13.17%), intestinal *Trichostrongylus* (7.59%) and intestinal *Impalaia* (4.73%) (Figure 3). A low fraction of ITS-2 profiles, corresponding to >0.1% of reads, was not assigned to the genus level.

Visual inspection (Figures 1 and 3) of diversity and proportions of strongylid genera for each gorilla group showed striking differences across Virunga Massif. In Volcanoes National Park (VNP) in Rwanda, the strongylid communities were dominated by *Murshidia* and *Hyostrongylus*, followed by other trichostrongylids. More specifically, *Hyostrongylus* dominated the gorilla groups in the southwest of VNP, where most gastritis cases have occurred, while *Murshidia* dominated towards the northeast, consequently meaning lower *Hyostrongylus* abundance among gorilla groups with fewer gastritis cases. In Virunga National Park (ViNP) in DRC, *Paralibyostrongylus*, which was essentially absent in VNP, and *Hyostrongylus* dominated the strongylid communities. The strongylid communities of the only gorilla group ranging in Mgahinga Gorilla National Park (MGNP) in Uganda, deviated from other Virunga Massif gorilla groups, being almost exclusively dominated by *Impalaia*. The absolute abundance of strongylid nematodes (relative abundance of strongylid genera combined with strongylid egg counts) was high in most VNP gorilla groups, with the highest abundance in *Murshidia*-dominated gorilla groups, while the absolute abundance was low in ViNP and MGNP gorilla groups (Figure 3), where clinical gastritis is yet to be observed.

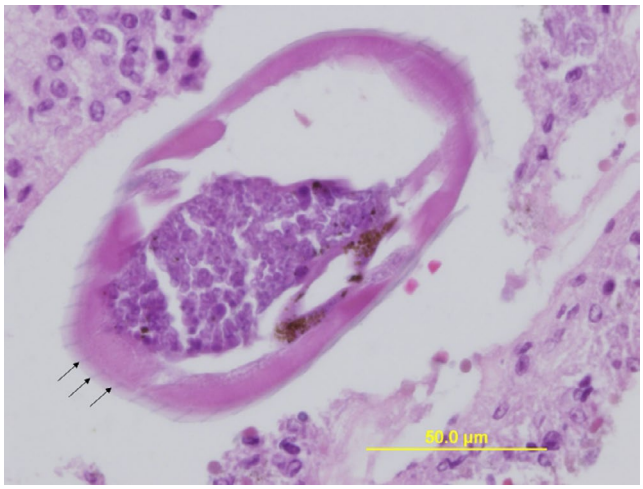


FIGURE 2 *Hyostrongylus* nematode observed in a gastric lesion during a histopathological examination (Fig. 3 from Muhangi et al., 2021, adjusted). The strongylid nematode was identified based on the presence of synlophe ridges (typical for the genus *Hyostrongylus*) indicated by arrows.

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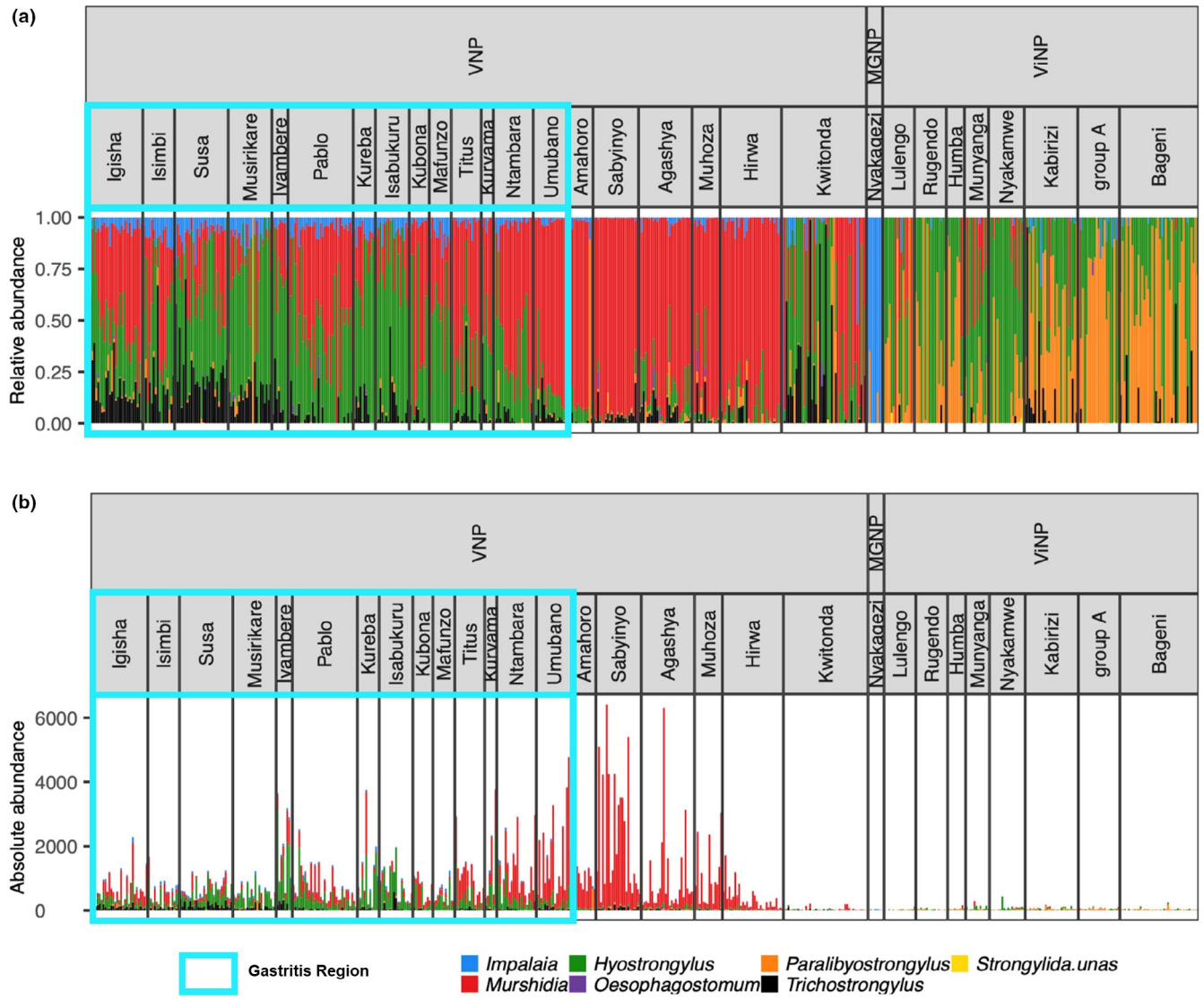


FIGURE 3 Bar plot showing the (a) relative abundance and (b) absolute abundance of strongylid genera in studied mountain gorilla groups inhabiting the Virunga Massif (Volcanoes National Park in northwestern Rwanda, Mgahinga Gorilla National Park in southwestern Uganda and Virunga National Park in eastern Democratic Republic of the Congo). The gastritis region, indicated by the blue box, highlights gorilla groups ranging within a single region where the majority (84%) of severe gastritis cases occurred.

We revealed that gorilla group identity explained 56.28% of total variation in relative abundance and 46.7% in absolute abundance of strongylid genera (PERMANOVA, $p=0.001$ in both cases). The strong effect of group identity was also evident based on PCoA ordination (Figure 4). In addition, the gorilla groups from VNP, ViNP and MGNP tended to cluster separately in the PCoA ordination space (Figure 4). Conversely, at the within-group level, sex/age and season explained only a low fraction of total variation in PERMANOVA models (<2%) despite being significant (Table 1).

With regards to the between-group predictors, using Redundancy Analysis, we showed that gorilla groups that had similar relative abundances of strongylid genera lived in geographic proximity with similar vegetation types (expressed as PC1 and PC2) and had similar core home range sizes, demonstrating how regionality of pathogenic *Hyostrongylus* dominated strongylid communities coincides with clinical case emergence. In the same analyses that accounted for differences in the absolute abundances of strongylid genera, only the effect of spatial proximity between groups was significant (Table 2).

To explore drivers of the individual most frequent strongylid genera, we applied Joint Species Distribution Models (JSDM) and revealed that the majority of strongylid genera (except for *Impalaia* and *Murshidia*) was affected by season and more commonly detected during the dry season in comparison to the wet season (Figure 5a). The presence of most genera (apart from *Hyostrongylus* and *Paralibyostrongylus*) was positively correlated with the PC2 axis for vegetation types, suggesting their occurrence is less frequent in mixed forest at lower elevations with less precipitation but higher temperatures. On the other hand, the PC1 axis affected the occurrence of only two strongylid genera, *Impalaia* (negative effect) and

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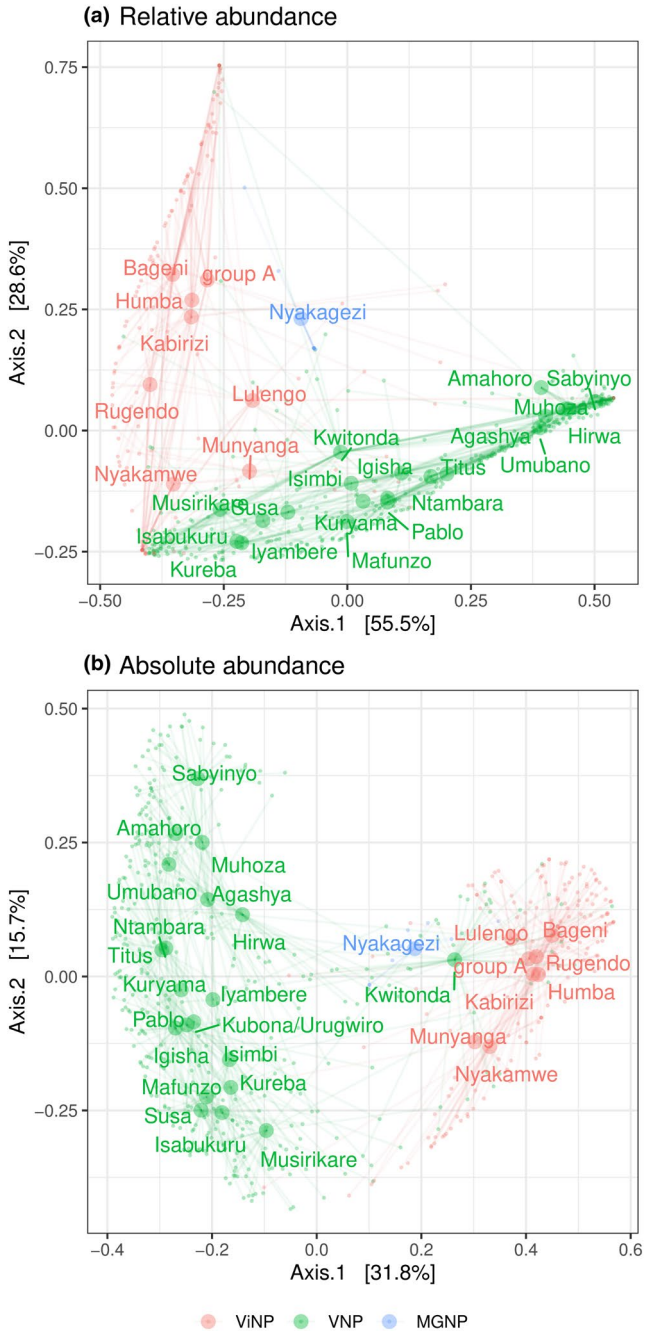


FIGURE 4 PCoA ordination depicting variation in (a) relative abundance and (b) absolute abundance of strongylid genera in mountain gorilla groups inhabiting the Virunga Massif (Volcanoes National Park in northwestern Rwanda, Mgahinga Gorilla National Park in southwestern Uganda and Virunga National Park in eastern Democratic Republic of the Congo, corresponding to the green, blue and red text, respectively). Lines connect each sample with centroids for a given gorilla group.

Paralibyostrongylus (positive effect), meaning *Impalaia* was more frequent in *Hypericum* woodland at higher elevations with more precipitation but lower temperatures, while the occurrence of *Paralibyostrongylus* decreased in this type of environment. Relative abundance of *Hyostrongylus* and *Paralibyostrongylus* were negatively affected by the PC2 axis (Figure 5b, Figure S2), implying their higher dominance in areas with high percentage of mixed forest at lower elevations with lower precipitation but higher temperatures. However, this effect was not detected when JSDM was run on absolute abundance of any strongylid genera (Figure 5c, Figure S3). Finally, core home range size had a positive effect on the occurrence of *Paralibyostrongylus* and negative effects on *Impalaia* and *Murshidia* (Figure 5a). We revealed no effect of gorilla density and group size on occurrences or relative and absolute abundance of any most frequent strongylid genus.

4 | DISCUSSION

Mountain gorillas remain vulnerable, existing in two isolated forest islands with relatively small population sizes (≈604 individuals in the Virunga Massif and ≈459 in Bwindi–Sarambwe; Granjon et al., 2020; Hickey et al., 2019). Despite remarkable conservation successes and a gradual increase in gorilla numbers in recent decades (Hickey et al., 2018), their restricted distribution and limited numbers mean that any factor increasing mortality rates and potentially slowing population growth or triggering a decline remains a serious cause for concern. In particular, the Virunga population lives in close proximity to some of the highest human population densities in Africa, which adds further pressure compared to the more sparsely populated regions surrounding western lowland gorilla habitat.

The mountain gorilla population in Virunga Massif has been facing an increasing number of serious or even fatal cases of gastrointestinal illness caused by strongylid nematodes (Muhangi et al., 2021). To understand the parasite-related gastrointestinal disease threatening this mountain gorilla population, we undertook an extensive survey of strongylid communities across the Virunga Massif population, utilizing both state-of-the-art and classical methods as recently recommended for parasitological studies (Ilík et al., 2024; Scholz, 2024). We identified *Hyostrongylus* nematodes in gastric lesions and reported pathogenic strongylid communities with increased absolute abundance of *Hyostrongylus* in the area where gastrointestinal illness occurred most frequently. Interestingly, *Hyostrongylus* is missing or rarely present in other studied asymptomatic populations of great apes in Africa (Ilík et al., 2023; Mason et al., 2022; Pafčo et al., 2018). *Hyostrongylus* infections in suids (e.g. domestic pigs, wild board and hogs) can have fatal consequences under certain conditions (Baskerville & Ross, 1970). Thus, we concluded that *Hyostrongylus* contributed to the development of gastrointestinal illness in the endangered Virunga Massif mountain gorilla population.

Overall strongylid communities in the Virunga Massif mountain gorilla population were dominated by gastric *Hyostrongylus* and *Paralibyostrongylus* and intestinal *Murshidia*, *Trichostrongylus* and *Impalaia*, which markedly differs from those of western lowland gorillas, despite their close phylogenetic proximity (Ilík et al., 2023; Mason et al., 2022; Pafčo et al., 2018). Strongylid communities in several populations of western lowland gorillas are consistently dominated by intestinal genera rare or absent in Virunga Massif gorillas, namely *Oesophagostomum* and *Necator*, with no serious health complications

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TABLE 1 Results of PERMANOVA analyses testing the effect of group identity and within-group factors on variation in relative and absolute abundance of strongylid genera in mountain gorillas in the Virunga Massif.

			Relative abundance			Absolute abundance		
Type	**Variable**	**Df**	R^2	*F*	*p*	R^2	*F*	*p*
Group identity	Group	27	0.51	20.216	0.001	0.407	13.342	0.001
	Residual	525	0.49			0.593		
Within group	Age/Sex	1	0.015	2.603	0.005	0.013	2.189	0.003
	Season	3	0.014	7.002	0.001	0.006	3.075	0.012
	Residual	501	0.972			0.981		

TABLE 2 Final db-RDA models including variables predicting strongylid genera community composition in mountain gorillas inhabiting the Virunga Massif.

Response	**Predictor**	**Df**	**Variance**	***F***	***p***
Relative abundance	PC2	1	0.071	4.758	0.018
	PCNM2	1	0.234	15.586	0.001
	PCNM3	1	0.113	7.562	0.004
	50% kernel home range size	1	0.06	4.007	0.024
	PC1	1	0.048	3.175	0.055
	Residual	22	0.33		
Absolute abundance	PCNM1	1	0.884	62.473	0.001
	PCNM2	1	0.118	8.345	0.008
	PCNM3	1	0.261	18.414	0.001
	Residual	24	0.34		

Note: The PCoA centroids for the community composition of each group were used as the RDA response. The final models in the table include only variables identified by the stepwise forward selection procedure. PCNM1, 2 and 3 represents spatial gradients between gorilla groups. PC1 is an environmental gradient positively associated with bamboo cover and negatively with *Hypericum*, PC2 is negatively associated with mixed forest. Models were built by forward selection procedure.

known in the wild western lowland gorillas (Ilík et al., 2023; Mason et al., 2022; Pafčo et al., 2018). Unlike western lowland gorillas, mountain gorillas occurring in isolated populations have historically experienced dramatic population declines and a severe bottleneck, reducing genetic diversity through small population sizes and several generations of inbreeding (Xue et al., 2015). We suggest that those events in mountain gorilla history could facilitate the emergence of new host–parasite interactions, with risk of health consequences in Virunga Massif gorillas, with the environment and possibly sympatric species playing an important role in those processes.

Strongylid nematodes are sensitive to vegetation and climatic conditions because of their direct life cycle and development. The eggs are shed in faeces and development to infectious filariform L3 larvae occurs within the external environment (Knapp-Lawitzke et al., 2016). The environmental conditions differ considerably between the habitats of western lowland gorillas and Virunga gorillas. In the Virunga Massif, altitudes range between 2000 and 3500 m a.s.l., with average temperatures from 0 to 10°C. In contrast, western lowland gorillas inhabit areas up to ~600 m a.s.l., where average annual temperatures remain above 23°C (Fick & Hijmans, 2017).

The strongylid nematodes infecting apes successfully complete environmental development in temperatures above 10°C and are capable of surviving several months below 4°C (Rose & Small, 1980, 1982). Though they favour higher temperatures (Agrawal, 1966; Fossing et al., 1995), their development (e.g. *Necator*) can cease below 17°C (Brumpt, 1958), explaining the absence of *Necator* in the strongylid communities of Virunga Massif gorillas. On the contrary, new strongylid communities could form through the adoption of strongylid taxa from sympatric hosts sharing the environment. *Hyostrongylus* is a typical parasite for suids and lagomorphs, commonly detected in Africa (Kouam & Ngueguim, 2022) but also in northern latitudes (Roepstorff & Murrell, 1997). Moreover, *Hyostrongylus* can also be transmitted to other grazing animals (Douvres & Tromba, 1958; Tromba & Douvres, 1958). *Murshidia* typically occurs in elephants (Chel et al., 2020) and *Impalaia* was originally described in impalas (Gibbons et al., 1977). Overall, the host spectrum for these strongylid genera is probably wider and not yet fully described, with parasitological studies of sympatric animals in Virunga Massif missing. Importantly, all these strongylids also occur in lowland gorillas, but with minimal representation, likely indicating incidental transmission events (Ilík et al., 2023; Mason et al., 2022; Pafčo et al., 2018), while in mountain gorillas these taxa have become dominant. Newly introduced parasites, particularly into small

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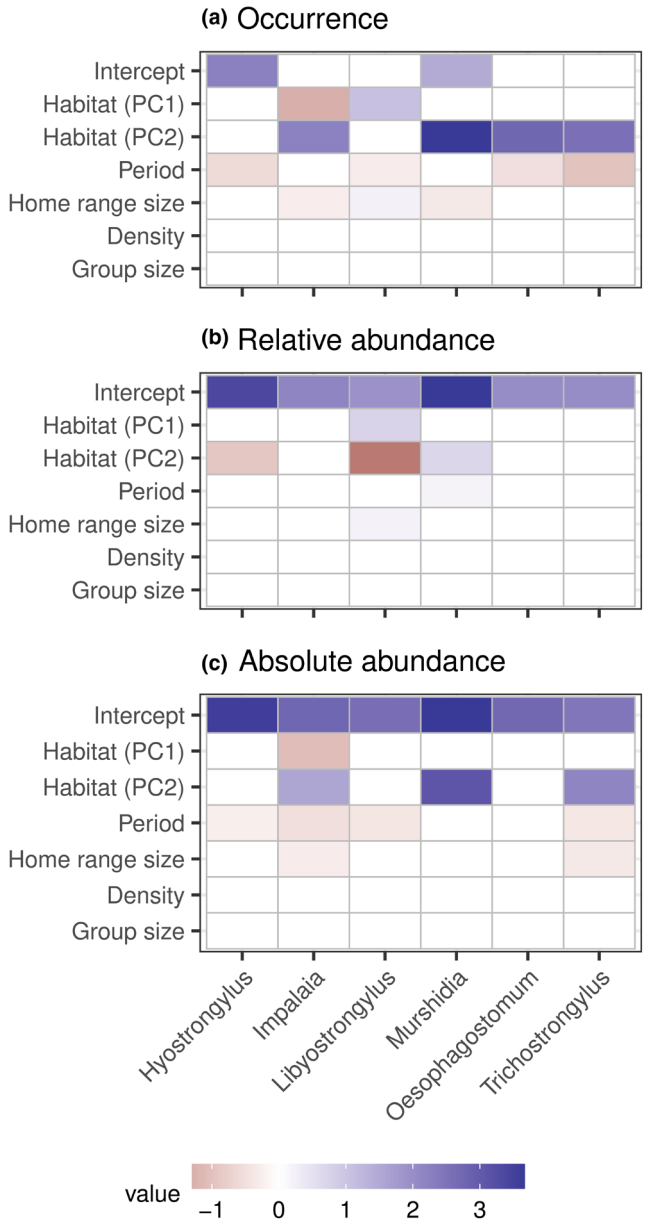


FIGURE 5 Heatmaps showing effect of predictor variables on strongylid genera (a) occurrence (presence/absence) (b) relative abundance and (c) absolute abundance in mountain gorillas inhabiting the Virunga Massif. Significant positive and negative associations that passed multiple testing corrections are indicated by blue and red colour, respectively.

host populations, are often characterized by higher pathogenicity (Holmes, 1996; Lymbery et al., 2014), as may be occurring in mountain gorillas, with the possibility of some detected strongylid taxa originating from either wild or domestic animals. With no buffer zone between the national park and surrounding agricultural fields, the introduction of new helminth infections into the gorilla population through spatial contact with domestic animals is entirely feasible, perhaps through gorillas visiting fertilized fields, especially on the Rwandan side. Importantly, none of the dominant strongylid taxa detected in Virunga gorillas have been reported as human parasites to date, further supporting the conclusion that these infections are not directly transmitted from humans but are more likely acquired from livestock or sympatric wildlife.

Strongylid communities of Virunga Massif gorillas lacked the uniformity observed across lowland gorilla populations (Mason et al., 2022), exhibiting a surprisingly high degree of variability within a single population. Although western lowland gorillas were sampled throughout their range (Mason et al., 2022), their strongylid communities were similar both within and between populations. According to WorldClim data (Fick & Hijmans, 2017), the habitats of studied western lowland gorillas are characterized by a relatively stable climate, with average temperature varying by 3°C between sites, elevation by several 100 m, and rainfall by a 100 mm/year, while differences within a single mountain gorilla population in the Virunga Massif are ten times greater. We showed that gorilla group identity, spatial segregation and vegetation/abiotic factors play a crucial role in strongylid community formation in Virunga Massif gorillas: gorilla groups ranging close to each other, occupying similar habitats and abiotic conditions with similar core home range size have analogous strongylid communities. This again points to the significant effect of environmental factors on the formation of strongylid communities in Virunga Massif gorillas, reflecting profound differences in vegetation and abiotic conditions observed across the relatively small area of Virunga Massif.

We distinguished three main types of strongylid communities in Virunga Massif gorillas (Figure 1), the first dominated by *Hyostrongylus* and *Paralibyostrongylus*, occurring in low absolute abundance in gorillas inhabiting low altitude with mixed forest and lower rainfall but higher temperatures. The second type was represented merely by the only gorilla group (Nyakagezi) inhabiting MGNP, characterized by mixed forest and bamboo, with communities dominated almost solely by *Impalaia*. The striking dominance of *Impalaia* is surprising. We speculate that the composition of Nyakagezi strongylid communities might be affected by the history of MGNP, as before the establishment of the national park the area underwent serious intensive illegal activities, with the original forest cleared for exotic plant agriculture until 1992, when it was left to naturally regenerate (Lejju et al., 2001). Such turbulent changes from high anthropogenic disturbance might alter strongylid communities. The third type of strongylid community is dominated by *Murshidia*, *Hyostrongylus* and other trichostrongylids in high absolute abundance in gorillas occurring in areas characterized by high elevations, with *Hypericum* woodland and bamboo, low temperatures and high precipitation.

On the Rwandan side of the Virunga Massif, clearance of the lowest vegetation zone (mixed forest) began in the late 19th and early 20th centuries, accelerating with the introduction of cash crops, such as potato and coffee. By the time Albert National Park (established in 1925, later divided into Virunga NP in DRC and Volcanoes NP in Rwanda) was created, much of Rwanda's lowland zone had already been converted to agriculture and thus excluded from protection (De Bont, 2017; Depicker et al., 2021). Additional excisions occurred in 1958 and again in 1969–73, further reducing the forest area (Weber & Vedder, 1983). As a result, mountain gorillas were confined to highest elevation habitats on the slopes and in the

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saddles of six volcanoes with cooler and wetter climatic conditions, which may represent suboptimal environments (Thorne et al., 2013). With the recent increase in gorilla group densities, a growing number of groups started spending prolonged time at higher elevations in the Virunga Massif (Eckardt et al., 2019) (55% of studied groups ranged between 2800 to 3450 m a.s.l.). Importantly, while this historical displacement set the stage for current ecological conditions, the clinical cases of strongylid-associated gastrointestinal disease have only been documented in recent decades. This suggests that a combination of long-term habitat restriction together with more recent increases in gorilla density, driven by conservation successes, may underlie the emergence of pathogenic strongylid communities rather than historical deforestation alone.

In this high-elevation region, characterized by *Murshidia*, *Hyostrongylus* and other trichostrongylids in high absolute abundance and challenging living conditions for gorillas, we further identified groups inhabiting the bamboo zone between Mt. Bisoke and Mt. Sabinyo. These groups exhibited the highest intensities of strongylid infections (EPG) across the Virunga Massif, with a dominance of intestinal *Murshidia*, which appears to be less or non-pathogenic. Only a limited number of gastritis cases were observed here, likely because of both low relative and absolute abundance of gastric *Hyostrongylus*. Secondly, groups with high relative and absolute abundance of gastric *Hyostrongylus* ranged in the highest elevations in *Hypericum* woodland between Mt. Karisimbi and Mt. Bisoke, where most of the gastritis cases occurred. High relative abundance of *Hyostrongylus* was also detected in gorillas ranging in ViNP, where no cases of gastritis were observed, but inclusion of egg counts revealed low absolute abundances of *Hyostrongylus* in these gorillas (see Figure 3). We thus conclude that the high absolute abundance of *Hyostrongylus* in the gorilla groups between Mt. Karisimbi and Mt. Bisoke at least partially, if not entirely, increased incidence of gastritis in gorillas in this area. This points to the absolute necessity of combining both quantitative (EPG) and qualitative (relative abundance) data in the research of emerging parasitological diseases. Moreover, identification of strongylid nematodes involved in gastric lesions from histopathological examinations further support *Hyostrongylus* as the cause, with the only other gastric strongylid, *Paralibyostrongylus*, virtually absent in the strongylid communities of gorillas between Mt. Karisimbi and Mt. Bisoke. Gastritis can be also caused by spiral bacteria (e.g. *Helicobacter*-like bacteria), but type of lesions caused by such bacteria were not found histologically in any of the cases or by molecular testing (Gorilla Doctors, data on file). Moreover, proliferative gastritis is known to be caused by other nematodes parasitic in stomach of various hosts, for example in cats—*Ollanulus tricuspis* and *Cylicospirura* sp., macaques—*Nochtia nochti* or cattle—*Ostertagia*. In pigs, *Hyostrongylus rubidus* attaches to the stomach mucosa and forms gastric nodules that lead to erosion and ulceration, causing gastritis in infected animals characterized by anaemia, anorexia and melena (De Witte et al., 2018; Dodd, 1960). Severity of the clinical symptoms of *Hyostrongylus* infection in pigs is impacted by several factors, for example the nutritional status or hormonal levels of the animal (Baskerville & Ross, 1970).

Although a previous study in Virunga Massif, which included unhabituated gorilla groups, associated overall strongylid intensities with the mean relative density of gorillas and size of their estimated home range (Petrželková et al., 2022), no impact of gorilla density was observed in the current study. Population surveys conducted between 2003 and 2016 across the entire Virunga Massif have revealed that population growth and gorilla spatial distribution have not been homogeneous across the landscape (Granjon et al., 2020). Between 1989 and 2010, gorillas in the area between Mt. Karisimbi and Mt. Bisoke had the highest population growth rates with the density in this area increasing at a higher rate than in the overall population (Caillaud et al., 2020), potentially gradually impacting parasite epidemiology in this area. As the last population census (2015–2016) revealed that the rest of the Virunga Massif population is currently experiencing similar growth rates as the area between Mt. Karisimbi and Mt. Bisoke did previously (Granjon et al., 2020), we cannot rule out shifts in parasite epidemiology and clinical manifestations affecting the other Virunga Massif subpopulations in the future. Thus, we recommend long-term monitoring of strongylid communities alongside population trends.

Our study underscores the ecological consequences of emerging health threats, such as strongylid nematode infections, in endangered host species, particularly in isolated populations like the Virunga Massif mountain gorillas. We show that populations living in potentially suboptimal environmental conditions can harbour diverse parasite communities, leading to significant health issues, including fatal disease. These emerging parasitic threats represent a serious conservation concern, with potential long-term effects on population viability. Additionally, the mountain gorilla's historical genetic bottleneck and reduced heterogeneity may contribute to a disrupted balance in host–parasite interactions, exacerbating the impacts of these infections. Our findings suggest that in highly managed wildlife populations, where population density increases within constrained habitats, the dynamics of parasitic diseases may begin to mirror those of livestock populations. This analogy calls for heightened monitoring and management strategies to ensure the continued success of conservation efforts. Because the Virunga gorillas live in close proximity to areas with high livestock densities, and the pathogenic strongylids identified in gorillas are well known from livestock, it will be important to systematically monitor domestic animals, especially pigs, characterize their strongylid communities and evaluate potential transmission patterns with gorillas. Given the widespread threat that strongylid nematodes pose to domestic animals (Hoste & Chartier, 1993; Zajac & Garza, 2020), it is crucial to recognize the potential for similar ecological and health risks in wildlife populations, particularly in areas impacted by anthropogenic pressures. By highlighting these emerging ecological challenges, our research emphasizes the need for interdisciplinary approaches in wildlife conservation, particularly in small, isolated populations. The findings from this study not only inform the conservation management of mountain gorillas but also provide valuable insights for managing the health of other endangered species that are similarly vulnerable to emerging diseases. Long-term ecological monitoring and a better understanding of host–parasite interactions in the context of changing

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environmental conditions will be critical to mitigating these threats and ensuring the survival of endangered species.

AUTHOR CONTRIBUTIONS

Barbora Pafco: Study design, laboratory work, drafting and editing the manuscript; Jakub Kreisinger: Bioinformatics and statistical analyses; Dusan Romportl: GIS analyses; Bethan Mason: Laboratory work, text improvement and English corrections; Barbora Cervena: Laboratory work; Carine Uwamahoro: Laboratory work and data curation; Winnie Eckardt: Field data collection and data curation, significant improvement of the manuscript; Kirsten Gilardi: Funding acquisition, significant improvement of the manuscript; Felix Ndagijimana, Julius Nziza, Eddy Kambale Syaluha, Bernard Ssebide: coordination of the fieldwork, field data collection and data curation; Richard Muvunyi, Prosper Uwingeli, Antoine Mudakikwa, Jeff Mapilanga, Arthur Kalonji, Robert Aruho: Coordination of the fieldwork; Jean Bosco Noheri, Methode Bahizi, Ricky Okwirokello, Fred Nizeyimana, Jean-Paul Lukusa, Jena Hickey: Field data collection and data curation; Jan Slapeta: Laboratory work; David Modry: Study design, laboratory work, funding acquisition; Linda Lowenstine: Histopathology observations; Hideo Hasegawa: Adult worm identification; Mike Cranfield: Study design, coordination of the field work and data curation; Klara J. Petrzelkova: Study design, coordination of the study, significant improvement of the manuscript, funding acquisition. All authors read and approved the manuscript.

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ACKNOWLEDGEMENTS

We are thankful to the Rwanda Development Board, the Uganda Wildlife Authority, the Uganda National Council of Science and Technology and Institut Congolais pour la Conservation de la Nature for their long-term support and for the permission to conduct this research. We are indebted to the field staff of the Dian Fossey Gorilla Fund, Gorilla Doctors, Rwanda Development Board, Virunga National Park, Institute Congolais pour la Conservation de la Nature, Uganda Wildlife Authority, and Mgahinga Gorilla National Park for their tireless support in collecting samples. We acknowledge the CF Genomics and the CF Bioinformatics supported by the NCMG research infrastructure (LM2023067 funded by MEYS CR) for their support with obtaining scientific data presented in this paper. Computation resources were provided by the e-Infrastruktura CZ Project (ID:90254), supported by the Ministry of Education, Youth and Sports of the Czech Republic. Open access publishing facilitated by Institute of Vertebrate Biology of the Czech Academy of Sciences, as part of the Wiley - CzechELib agreement.

FUNDING INFORMATION

This work was supported by the Czech Science Foundation, grant no. 18-24345S and 22-16475S and by the U.S. Fish and Wildlife Service Division of International Affairs, grant no. F17AP00964 and by institutional support from the Institute of Vertebrate Biology, Czech Academy of Sciences (RVO:68081766) and Masaryk University (MUNI/A/1602/2023).

CONFLICT OF INTEREST STATEMENT

Authors declare that they have no competing interests.

DATA AVAILABILITY STATEMENT

Raw sequencing data are archived in the European Nucleotide Archive under the accession number of the whole project PRJEB91131 (available at: https://www.ebi.ac.uk/ena/browser/view/PRJEB91131) (Pafco et al., 2025). Sample-specific accession numbers are provided in Table S3.

STATEMENT ON INCLUSION

This research involved collaboration of scientists working in multiple countries, with collaborators from all surveyed localities included as co-authors. The research addresses valuable questions, and the results will be shared with all research teams involved as well as the wider scientific community.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

Figure S1. PCA biplot for percentage vegetation type components within 50% kernel home ranges of individual social groups.

Figure S2. Plot showing effect of predictor variables on strongylid nematode relative abundance in mountain gorillas inhabiting the Virunga Massif.

Figure S3. Plot showing effect of predictor variables on strongylid infection intensities in mountain gorillas inhabiting the Virunga Massif.

Table S1. Number of samples and mountain gorilla groups inhabiting the Virunga Massif: Volcanoes National Park (VNP) in northwestern Rwanda, Mgahinga Gorilla National Park (MGNP) in southwestern Uganda and Virunga National Park (ViNP) in eastern Democratic Republic of the Congo, included in the present study.

Table S2. Pearson correlations between first two PCA gradients in vegetation type structure and abiotic factors characterizing 50% kernel home ranges social groups.

Table S3. Metadata for mountain gorilla groups inhabiting the Virunga Massif, including Volcanoes National Park (VNP) in northwestern Rwanda, Mgahinga Gorilla National Park (MGNP) in southwestern Uganda, and Virunga National Park (ViNP) in eastern Democratic Republic of the Congo. The table also provides ENA (The European Nucleotide Archive) accession numbers for the raw sequencing data corresponding to each gorilla sample included in this study.

How to cite this article: Pafco, B., Kreisinger, J., Romportl, D., Mason, B., Cervena, B., Uwamahoro, C., Eckardt, W., Ndagijimana, F., Nziza, J., Kambale Syaluha, E., Ssebide, B., Noheri, J. B., Bahizi, M., Okwirokello, R., Nizeyimana, F., Lukusa, J.-P., Gilardi, K., Muvunyi, R., Uwingeli, P., … Petrzelkova, K. J. (2026). Emerging pathogenic strongylid infections in Virunga Massif mountain gorillas: Role of environmental stressors. *Journal of Applied Ecology*, *63*, e70258. https://doi.org/10.1111/1365-2664.70258

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Mason, B., Ilík, V., Červená, B., Cranfield, M., Nziza, J., Ssebide, B., Gilardi, K., Uwamahoro, C., Eckardt, W., Ndagijimana, F., Muvunyi, R., Uwingeli, P., Mudakikwa, A., Aruho, R., Whittier, C.A., Kreisinger, J., Samaš, P., Nosková, E., Modrý, D., Pafčo, B., Petrželková, K.J.

2025

Untangling parasite epidemiology of mountain gorillas through historical samples: strongylid nematodes are friends or foe?

Biol. Conserv. 310, 111319. https://doi.org/10.1016/j.biocon.2025.111319

Biological Conservation 310 (2025) 111319



Contents lists available at ScienceDirect

Biological Conservation

journal homepage: www.elsevier.com/locate/biocon





Untangling parasite epidemiology of mountain gorillas through historical samples: Strongylid nematodes are friends or foe?

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ARTICLE INFO

Keywords:
Island populations
Biobank
Wildlife disease
Primate
Helminth
Hyostrongylus
Metabarcoding

ABSTRACT

Continued ecosystem changes create a dynamic ensemble of threats for wildlife species of conservation concern, including emergence of novel pathogens, a prominent risk amongst isolated populations with lowered resilience. Success in the conservation of mountain gorillas (*Gorilla beringei beringei*) is owed to intensive stewardship over the last three decades, with ongoing management required in the face of continued global change. Recently, increased clinical disease occurrence is reported amongst the only two extant mountain gorilla populations, with symptoms typical of strongylid nematode infections. Using historic biobanked faecal samples, we assessed changes in the strongylid nematode communities of both isolated mountain gorilla populations between two decades, employing high-throughput sequencing to describe their genetic diversity and explore their relevance to clinical case emergence. We applied ITS-2 metabarcoding within the Illumina MiSeq platform for strain-level identification. We uncover major temporal variability of strongylid communities in the region with the highest disease occurrence, revealing that dominance of pathogenic *Hyostrongylus* has emerged within this region within the last two decades. This coincides with considerable changes in gorilla social structure in this region since 2007, following continuous population growth but limited ability for spatial expansion. The recovery of mountain gorillas provides a valuable lesson to conservation science yet highlights how ongoing growth of any island populations requires vigilant management to ensure resilience. Emerging threats, such as novel diseases, must be carefully considered within these management plans, with biobanking and long-term monitoring a vital conservation tool for preventing biodiversity loss.

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https://doi.org/10.1016/j.biocon.2025.111319
Received 11 November 2024; Received in revised form 10 June 2025; Accepted 15 June 2025
Available online 8 July 2025
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8.26

Sambucci, K.M., Samaš, P., Ssebide, B., Petrželková, K.J., Okello, R.O., Nizeyimana, F., Bukamba, N., Smiley-Evans, T., Gilardi, K., Pafčo, B., Červená, B.

2026

Shifts in strongylid communities associated with chronic wasting in mountain gorillas.

Int. J. Parasitol. 56, 104848. https://doi.org/10.1016/j.ijpara.2026.104848

International Journal for Parasitology 56 (2026) 104848

Contents lists available at ScienceDirect

International Journal for Parasitology

journal homepage: www.elsevier.com/locate/ijpara

Shifts in strongylid communities associated with chronic wasting in mountain gorillas

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ARTICLE INFO

Article history:
Received 19 November 2025
Received in revised form 24 March 2026
Accepted 27 March 2026
Available online 2 April 2026

Keywords:
Strongylid nematodes
Metagenomics
Mountain gorilla
Oesophagostomum
Chronic wasting

ABSTRACT

Host–parasite relationships are typically maintained in a dynamic equilibrium, but disruptions to this balance can lead to clinical disease and population-level health impacts. Chronic wasting, characterized by chronic loss of body condition, alopecia, a browning hair coat and pot belly, is an emerging health concern in mountain gorillas of Bwindi Impenetrable National Park, Uganda. Deworming of suspected cases has led to marked short-term health improvements, implicating intestinal helminths. To investigate, we analysed faecal samples from human-habituated gorillas collected in 2018 and 2021, and unhabituated gorillas in 2018, using high-throughput sequencing of strongylid nematodes (ITS-2) and gut bacteria (16S). Strongylid community composition varied with chronic wasting occurrence, with *Oesophagostomum* emerging as a key taxon driving this difference, while bacterial communities remained relatively stable. Strongylid diversity increased between 2018 and 2021, and habituated gorillas exhibited reduced strongylid genetic diversity, higher relative abundance of *Oesophagostomum* and lower relative abundance of *Murshidia* compared to unhabituated gorillas. These results suggest that a higher abundance of *Oesophagostomum* is associated with chronic wasting in mountain gorillas due to either a causative association or other genetic, immunological or environmental causes allowing *Oesophagostomum*, a common member of the gut eukaryote community of the Bwindi gorillas, to overpopulate.
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1. Introduction

Host–parasite relationships are shaped by long-term coevolution, which can result in relatively stable associations under certain ecological conditions, although the fitness costs to hosts can vary widely (May and Anderson, 1990). However, environmental change, altered host ecology, or anthropogenic disturbance can disrupt this balance, shifting parasites from commensal or subclinical states to agents of disease (Patz et al., 2000). Such imbalances can have profound consequences for individual health, population viability, and even species conservation, particularly in long-lived or small populations (Russell et al., 2020). Non-human primates are no exception, with parasitic infections contributing to morbidity and, in some cases, mortality in both free-ranging and captive populations (Jaffe et al., 2024; Ngoubangoye et al., 2021; Terio et al., 2018).

The mountain gorilla (*Gorilla beringei beringei*) is the only great ape species increasing in number, with a most recent population

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https://doi.org/10.1016/j.ijpara.2026.104848
0020-7519/© 2026 The Author(s). Published by Elsevier Ltd on behalf of Australian Society for Parasitology.
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estimate of 1,063 individuals (Hickey et al., 2019), thanks to intensive conservation efforts which include habitat preservation, anti-poaching patrols and hands-on veterinary care provided by Gorilla Doctors (Robbins et al., 2011). Despite this success, they remain vulnerable due to their small population size, low genetic diversity, and risk of emerging infectious diseases (Xue et al., 2015). Mountain gorillas exist in two isolated populations; one in the Virunga Massif spanning Rwanda, Uganda and the Democratic Republic of the Congo (DRC), and the other in the Bwindi-Sarambwe Ecosystem, encompassing Bwindi Impenetrable National Park (BINP) in Uganda and the Sarambwe Nature Reserve in DRC (IUCN, 2018).

In recent years, chronic wasting cases have increased in habituated Bwindi gorillas. The condition is characterised by weight loss, lethargy, and poor hair coat, including browning and patchy alopecia (Fig. 1). Post-mortem examinations revealed high nematode burdens, and anthelmintic treatment led to recovery in some individuals, suggesting an etiologic role of intestinal helminths. It remains unknown whether unhabituated gorillas are similarly affected, as their health cannot be routinely monitored. Habituation is associated with increased disease transmission risk and potential shifts in pathogen epidemiology due to closer human-gorilla interactions and ecological changes (Shutt et al., 2014).

Intestinal helminths have long been studied in mountain gorillas, from early work in Rwanda in the 1970s (Redmond, 1983), to classical parasitological surveys in Bwindi (Ashford et al., 1990; Rothman et al., 2008; Sleeman et al., 2000). Only recently have helminth communities been compared across mountain gorilla populations (Petrželková et al., 2021), prompted by increasing reports of gastrointestinal disease. This work revealed substantial spatial heterogeneity in strongylid infections, with gorillas in BINP exhibiting higher strongylid egg counts than those in the Virunga Massif (Petrželková et al., 2021), a pattern also observed historically (Ashford et al., 1996; Rothman et al., 2008; Mason et al., 2025). Within Virunga, strongylid egg counts varied markedly across sites and were highest in areas with elevated gastritis prevalence; these patterns were linked to environmental factors such as altitude, rainfall, and vegetation type rather than habituation status (Petrželková et al., 2021, 2022). In Bwindi, no obvious patterns for strongylid egg counts were observed (Petrželková et al., 2021). Subsequent high-throughput sequencing (HTS) studies further refined these associations by identifying nematodes of the genus *Hyostrongylus*, gastric strongylids known to cause pathology in suids (Baskerville and Ross, 1970), as highly abundant in Virunga gorilla groups experiencing the most severe gastritis (Mason et al., 2025; Pafčo et al., 2026). Moreover, longitudinal analyses demonstrated that *Hyostrongylus* abundance has increased substantially over the past two decades in the same regions where gastritis incidence has risen (Mason et al., 2025). Together, these findings indicate that shifts in strongylid community composition, rather than infection intensity alone, are closely associated with gastrointestinal pathology in mountain gorillas. In contrast, the tapeworm *Anoplocephala gorillae,* common in both Bwindi and Virunga populations, does not seem to impair host health in most cases (Červená et al., 2024; Muhangi et al., 2021; Petrželková et al., 2021, 2022).

In the present study, we apply an integrated HTS-based approach, previously used to identify the parasitic cause of gastritis in Virunga mountain gorillas (Mason et al., 2025; Pafčo et al., 2026), to investigate strongylid nematode and bacterial communities in Bwindi gorillas. Strongylid nematodes can be associated with pathological changes in non-human primates (Jaffe et al., 2024; Pafčo et al., 2026; Terio et al., 2018) and are suspected in chronic wasting, while gut bacteria are recognized as key modulators of host health (Hou et al., 2022). Given the bacteriome's central role in immune regulation, it may influence both susceptibility to and the course of helminth infections and associated pathologies (Zaiss and Harris, 2016), including chronic wasting. Against this background, we investigate whether strongylid nematodes and gut bacteria are associated with the occurrence of chronic wasting in Bwindi gorillas, and whether they have contributed to the observed increase in chronic wasting cases over time. We also compare gorilla groups based on their home range position within the forest: those that range near the forest edge versus those that spend their time entirely within the national park. This distinction was chosen due to the large disturbance and lack of buffer zone between the park and community land.



Fig. 1. Photograph of adult female gorilla showing the chronic wasting clinical symptoms on left and on right back of healthy adult female gorilla for comparison.

The area surrounding the national park is densely populated largely with agriculturalists from the Bakiga and Bafumbira ethnolinguistic groups, in addition to Batwa who traditionally resided in the forest as hunter-gatherers until 1991 (Tumusiime et al., 2018). Although access to the forest is formally restricted to rangers, trackers, tourists, and researchers, mountain gorillas frequently range beyond park boundaries into community land, increasing their exposure to human-modified environments (Seiler and Robbins, 2016).

Altogether, in our analyses we specifically assess whether strongylid community composition, helminth burden, or host-related factors are associated with chronic wasting, and whether this varies over time. We also examine whether strongylid communities differ between habituated and unhabituated gorillas or based on the gorilla group forest position to evaluate potential influences of daily human proximity. Finally, we investigate whether gut bacterial community structure covaries with chronic wasting occurrence, helminth burden, or ecological factors, and whether specific bacterial taxa are associated with chronic wasting.

2. Materials and methods

2.1. Study site

The study was conducted in Bwindi Impenetrable National Park (BINP) located in Southwest Uganda, 01°01′S 29°41′E/1.017°S 29.683°E, an Afromontane lowland forest extending into the montane forest belt with elevations from 1,160 to 2,607 m (UNESCO, 2023). The forest is characterised by a complex mosaic of vegetation types, including lowland and montane forest, bamboo zones, and forest edges, shaped by the steep altitudinal gradient (Babaasa et al., 2024). A total of 459 gorillas were recorded in the most recent census (Hickey et al., 2019). The area surrounding BINP is densely human-populated (100–450 individuals/km^2) with limited to no buffer zone (BirdLife International, 2025). BINP experiences biannual rainy seasons with long rains from March to June and short rains from September to November (Ssentongo et al., 2018).

2.2. Sample collection and sample sets

This research involved only non-invasive work with wild non-human primates. All work was approved and conducted in collaboration with the Uganda Wildlife Authority (COD/96/05), the Uganda National Council of Science and Technology (NS644; HS2416ES), and undertaken in accordance with their respective rules and regulations. Field site access was facilitated by in-country collaborators with access permissions and long-term research activities, all of whom are included as co-authors.

Fresh, unidentified faecal samples from habituated gorillas were collected non-invasively from night nests in BINP during two sampling periods: September–October 2018 and October 2021. Additionally, ethanol-preserved faecal samples from the 2018 census collected between March and May (Hickey et al., 2019) were available. In total 391 mountain gorilla faecal samples were used: 296 from habituated gorillas (across 2018 and 2021) and 95 from unhabituated gorillas (2018 only). Bacterial 16S rRNA gene data were available for 151 habituated gorilla samples from 2021. Samples of habituated gorillas were collected from 10 groups in both 2018 and 2021, from 3 groups in 2018 only and from 7 groups in 2021 only, giving 13 groups sampled in 2018 and 17 groups in 2021. The samples were collected from each group only once and as gorillas defecate in their own nest leading to one faecal portion present, unless it is a mother and infant and the difference in faecal size is obvious, the risk of the same individual being sampled more than once is negligible.

Faecal samples were homogenized and ~1 g preserved in 96% ethanol and stored at ambient temperature for several weeks before they were transported to the laboratory in the Czech Republic. Upon arrival, samples were stored at 4°C or −20°C until DNA extraction. Storage duration before DNA isolation ranged from several weeks to months for the 2018 and 2021 samples, and several years for the census samples. In habituated gorillas, an aliquot of fresh sample was examined within 48 h of estimated defecation by microscopy, and strongylids and tapeworms were quantified using Mini-FLOTAC© to calculate eggs per gram of faeces (EPG) (Modrý et al., 2018). Fresh samples were unavailable for unhabituated gorillas and their formalin-preserved aliquots were stored for a long time prior to examination, therefore, because of the potential disintegration of the eggs over time, we could not compare faecal egg counts between the habituated and unhabituated gorillas.

For parasitological examination, approximately 3 g of faeces were processed following the protocol described in Petrželková et al. (2021). Samples were homogenized in water, strained, centrifuged (2,500 rpm, 5 min), and the sediment was weighed and resuspended in saturated NaCl (specific gravity 1.2) to a total volume of 45 mL. Egg counting was performed using the Mini-FLOTAC device by filling both chambers and counting eggs after 10 min under 10× magnification. Counts were converted using the Mini-FLOTAC multiplication factor (eggs counted in two chambers × 7.5), corresponding to a detection limit of 8 EPG. Weighing the sediment enabled us to calculate eggs per gram of sediment (EPGS). This was obtained by multiplying the egg count in the two chambers by 22.5 (to estimate the total number of eggs in the 45 mL suspension) and dividing by the sediment weight. In 2021, a subset of samples was preserved in formalin and analysed within one month using a sedimentation method. After centrifugation, the sediment was weighed, resuspended to 10 mL, and a 0.2 mL aliquot examined microscopically. Egg counts were converted using formula EPGS = egg count × 50/sediment weight. Therefore, all results are reported as eggs per gram of sediment.

Chronic wasting cases were compiled for 2018–2022 from the records of Gorilla Doctors, a US-based NGO with an international team of Rwandan, Ugandan, and Congolese veterinarians responsible for health monitoring and veterinary care of eastern gorillas. Park rangers visit habituated gorillas daily and visually report on every individual in the group. They are trained to recognize any early signs of chronic wasting including rough or discoloured hair coat, weight loss and/or pot bellies and report these signs to Gorilla Doctors for an immediate veterinary monitoring check. A Gorilla Doctors veterinarian assigns a classification of chronic wasting based on the following criteria: an adult gorilla exhibiting generalized observable weight loss, as evidenced by loss of muscle mass around the limbs, ribs, loins, back, shoulders, head and most body parts of approximately 10–20% of its estimated body weight, over a minimum period of 10 weeks, or an infant gorilla that is failing to gain weight and grow, and which is also experiencing at least one of the following clinical signs: (i) dull-coloured hair-coat with loss of the shiny black hair colour; (ii) brownish hair-coat colour progressive appearance over the course of time; (iii) hair loss on the shoulders, nape, back, limbs, wrists, perineum, inguinal areas leaving a whitish or pale skin; (iv) matting of hair around anus and perineum, constipation, abnormal faeces: diarrhoea, abnormal colour of faeces, presence of mucous and/or grossly observable live helminths or proglottids in faeces; (v) bloated or distended or sagging abdomen (young individuals); (vi) loss of appetite; (vii) apathy. It should be noted that signs of chronic wasting are non-specific and can mimic other forms of non-parasitic systemic disease including but not limited to micronutrient deficiencies, chronic organ system conditions (i.e., kidney or liver disease) and poten-

tially chronic stress. Our case definition did not differentiate between these potential causes and was designed as an introductory investigation of potential factors associated with this syndrome.

Two representations of chronic wasting occurrence were used depending on the analytical approach. For alpha diversity analyses, the continuous chronic wasting case count was used – defined as the total number of symptomatic individuals per group across the sampling year and the immediately following year, the latter included to account for the possible delay between strongylid infection and clinical manifestation (Jürgenschellert et al., 2022). This continuous predictor was used alongside group size as a simultaneous covariate, which adjusts for variable group sizes without losing information through categorisation. For beta diversity and differential abundance analyses, chronic wasting prevalence was calculated as the proportion of affected individuals within each group (number of chronic wasting cases/group size), using the same time window. Groups were then classified into three ordered chronic wasting burden categories: low (≤5% prevalence), moderate (>5–15%), and high (>15%). Categorisation was preferred over a continuous prevalence value in these analyses for three main reasons: it allows explicit pairwise contrasts between burden levels that are directly interpretable; it does not assume a strictly linear relationship between chronic wasting burden and community composition, which is unlikely across the full prevalence range; and it aligns the statistical grouping directly with the visual ordination, facilitating interpretation. Thresholds were chosen to reflect the distribution of prevalence values in the dataset while maintaining broadly comparable sample sizes across categories.

For unhabituated gorillas, GPS position at the sample collection site was used as a location proxy. For habituated gorillas GPS positions for each group and data on group size (number of individuals) were obtained from Uganda Wildlife Authority databases for 2018 to 2021 with a monthly frequency. For further analyses of the chronic wasting occurrence in habituated gorillas, monthly averages of group size were further aggregated to annual averages, resulting in one group size value per year for each habituated group sampled. To reflect the different levels of anthropogenic pressure, habituated groups were further classified by forest position – those groups that spent time outside the park borders were categorized as edge (10 groups), while those that remained entirely within the forest were classified as inside (10 groups).

2.3. DNA isolation and sequencing

Samples were incubated at 37°C overnight to evaporate ethanol before DNA isolation. DNA was extracted using DNeasy® PowerSoil® Pro Kit (QIAGEN, Hilden, Germany), adapted with a 30-minute bead-beating step and a two-step elution (2 × 50 µl). A two-step-PCR using amplicon specific primers in the first PCR and Nextera primer design in the second PCR was used, followed by Illumina MiSeq sequencing. Strongylid ITS-2 was amplified with the forward primer Strongyl_ITS-2_F (5′-ACGTCTGGTTCAGGGTTG-3′) and the reverse Strongyl_ITS-2_R (5′-ATGCTTAAGTT CAGCGGGTA-3′) (Pafčo et al., 2018, 2019), while to target the bacterial 16S rRNA gene (V3–V4 regions) primer pair S-D-Bact-0341-b-S-17 (5′-CCTACGGGNGGCWGCAG-3′) and S-D-Bact-0785-a-A-21 (5′-GACTACHVGGGTATCTAATCC-3′) was used (Klindworth et al., 2013; Mason et al., 2022). All samples had technical duplicates with negative and positive controls.

2.4. Bioinformatics

Gene-specific primers were trimmed using SKEWER (Jiang et al., 2014). Paired-end reads were merged and denoised with DADA2 (Callahan et al., 2016) removing sequences with an expected errors above 2. To prevent inflated diversity from residual PCR or sequencing errors, only Amplicon Sequence Variants (ASVs) detected in both technical replicates were retained (Pafčo et al., 2018). Taxonomic assignments were performed using the naïve Bayesian RDP classifier within the DADA2 pipeline v 1.30.0 in R version 4.3.2 (R Core Team 2023). For strongylid ASVs, a custom ITS-2 reference database was built by combining sequences generated in our laboratory with the top 100 BLASTN hits from the NCBI nucleotide database (excluding environmental samples), whereas bacterial ASVs were classified against the SILVA 16S rRNA database v 138.1 (McLaren and Callahan, 2021). ASVs lacking genus-level (Strongylida) or phylum-level (bacteria) assignment were excluded. Sequence data and metadata were integrated in a phyloseq object using phyloseq package v.1.41.1; (McMurdie and Holmes, 2013).

2.5. Statistical analyses

Analyses were conducted using R version 4.5.0 (R Core Team, 2025). Association between chronic wasting occurrence and strongylid communities were tested alongside host (group size) and environmental (forest position, sampling year) factors. Chronic wasting occurrence was represented as a continuous covariate (number of cases) in alpha diversity models and as an ordered categorical variable (low/moderate/high prevalence) in beta diversity and differential abundance analyses, as explained in Section 2.2. To assess habituation effect, strongylid communities were compared between habituated (2018) and unhabituated (2018 census) samples. Effects of chronic wasting occurrence and environmental factors on gut bacterial community composition were also analysed for 2021 habituated samples.

For both strongylid and microbiome data, differences in sequencing depth between samples were corrected by standardising counts to relative abundances (the proportion of each taxon within a sample). For strongylid analyses in habituated gorillas, we also incorporated infection intensity by combining faecal egg counts (EPGS) with relative abundances. This produced "absolute abundance" profiles, reflecting the estimated number of eggs per gram of faecal sediment contributed by each genus. We ran parallel analyses using relative abundances alone, and these yielded congruent results.

2.5.1. Alpha diversity analyses

Effects of chronic wasting case counts, sampling year (2018 vs. 2021), group size, forest position (forest interior vs. edge), tapeworm EPGS, and strongylid EPGS on strongylid alpha diversity (outcome variables) were evaluated using three metrics: genus-level haplotype richness (counts), Shannon diversity, and Simpson diversity (both continuous). For strongylid richness, a generalized linear mixed model (GLMM) with a negative binomial distribution was used. The Shannon diversity index was exponentially transformed to achieve a normal distribution of model residuals, and both this index and the inverse Simpson diversity index (1/D) were evaluated using linear mixed models (LMMs) with Gaussian distribution. Gorilla group identity was included as a random intercept in all models to account for repeated sampling. All mixed models were fitted as full models containing all predictors simultaneously. Statistical significance of individual predictors was assessed using Type III Wald tests within the full model, thereby estimating the marginal contribution of each predictor while accounting for the others. For bacterial data, alpha diversity was calculated from the ASV table, whereas taxonomic composition was summarized at the phylum level for visualisation, beta-diversity ordination, and differential abundance analyses. Sampling year was excluded from bacterial models due to single-year sampling.

2.5.2. Beta diversity analyses

Variation in strongylid community composition (beta diversity; outcome variable) in relation to chronic wasting prevalence category, group size, sampling year and forest position predictors were examined using absolute abundance data. Community composition was visualised using Principal Component Analysis (PCA) of Aitchison distances, computed as Euclidean distances on centred log-ratio (CLR) transformed counts, with zeros replaced by half the within-sample minimum non-zero value prior to transformation. Statistical significance of predictors was assessed using PERMANOVA-S implemented via adonis2 function in vegan v.2.6-4 (Oksanen et al., 2022) with marginal tests and blocked permutations that restricted permutations within groups, thereby accounting for group-level pseudoreplication. However, blocked permutations require that the predictor of interest varies within at least some groups – that is, that some groups contain individuals from more than one category. This condition was not met in two of our analyses. In the habituation comparison, all individuals within a group share the same habituation status by definition. In the bacteriome analysis, chronic wasting prevalence category is assigned at the group level, meaning all individuals within a group belong to the same category. In both cases, blocked permutations cannot reassign labels across groups. Results for these comparisons are therefore presented descriptively using PCA ordination and multivariate dispersion testing (betadisper, vegan), which assesses whether groups differ in within-group spread rather than centroid location and are not subject to the same constraint. To test robustness to potential confounding between sampling year and group composition in strongylid community, we conducted a sensitivity analysis restricting data to the 10 groups sampled in both 2018 and 2021 (n = 187 samples). The same approach was used to examine bacterial community composition, utilizing relative abundance data at phylum level, excluding the temporal effect of sampling year.

To identify specific strongylid and bacterial taxa whose abundances were associated with chronic wasting, Analysis of Compositions of Microbiomes with Bias Correction (ANCOM-BC2) was applied using the three ordered chronic wasting prevalence categories defined in Section 2.2 (low ≤5%, moderate >5–15%, high >15%) using the ANCOMBC package (v2.4.0) (Lin and Peddada, 2020). This log-linear regression model addresses key challenges in analysis of HTS data, including compositionality, zero inflation, and variance regularization, while explicitly controlling for confounding variables of forest position (for strongylid and bacteria models), group size and sampling year (for strongylid model only). In addition to standard statistical outputs, the Log-Fold Change (LFC) was provided by the model, which quantifies the magnitude of change in taxa abundance between groups on a natural log scale. LFC values about or greater than ±1.5 (representing >4-fold changes in abundance) were considered biologically meaningful, as smaller fold-changes may be statistically significant but too minor to have practical biological importance (McCarthy and Smyth, 2009). Strongylid differential abundance analysis was performed using the absolute abundance data. As chronic wasting prevalence is assigned at the group level, all samples within a group share the same chronic wasting prevalence category, meaning that ANCOM-BC2 does not fully account for the non-independence of samples nested within groups. This pseudoreplication may inflate type I error rates for the chronic wasting effect. To provide a more conservative and statistically rigorous test of our a priori hypothesis regarding *Oesophagostomum* association with chronic wasting, we additionally fitted a genus-specific GLMM with group identity as a random intercept and a negative binomial error distribution. By explicitly modelling between-group variance as a random effect, this approach treats the gorilla group, rather than the individual sample, as the effective unit of replication for the group-level chronic wasting predictor. Results of this GLMM are therefore treated as the primary evidence for the *Oesophagostomum*–chronic wasting association, with ANCOM-BC2 results serving as a complementary screen across all genera.

Finally, using relative abundance data (as absolute EPGS-corrected abundance was unavailable for unhabituated groups), the same analytical framework described above was employed to compare strongylid communities between habituated and unhabituated gorillas (using 2018 data only), including diversity analyses and ANCOM-BC2 testing.

Covariation between strongylid and bacterial communities was assessed using two complementary approaches, both restricted to habituated individuals sampled in 2021 for which matched strongylid ITS-2 and bacterial 16S rRNA gene data were available (N = 151). Both ASV tables were first agglomerated to genus level and CLR-transformed using a half-minimum replacement for zeros to reduce compositionality bias prior to correlation analyses. At the community level, overall covariation between strongylid and bacterial communities was assessed using a Mantel test (Spearman correlation, 999 permutations) on Aitchison distance matrices computed from the CLR-transformed genus-level tables. At the genus level, pairwise Spearman correlations were computed between all strongylid genera and all bacterial genera retained at ≥10% prevalence across samples. P-values were adjusted across all pairwise tests using the Benjamini–Hochberg false discovery rate procedure and associations with $q < 0.05$ were considered significant.

3. Results

Chronic wasting cases increased from 8 in 2018 to 30 in 2022, with the total number of 67 cases recorded between 2018 and 2022 (Table S1). In groups sampled in this study, chronic wasting prevalence ranged from 0% to 27% across groups, which were subsequently classified into three prevalence categories: low (≤5%; 105 samples from 10 groups), moderate (5–15%; 59 samples from 6 groups), and high (>15%; 99 samples from 10 groups).

3.1. Strongylid communities differed by chronic wasting prevalence category

All faecal samples contained at least one helminth with strongylid egg counts (EPGS) ranging from 9 to 12,453 in 2018 and 49–12,453 in 2021 and tapeworm EPGS ranging from 17 to 2,806 in 2018 and 0–4,143 in 2021 (Table S2).

Sequencing of the strongylid ITS-2 region yielded 13,773,448 high-quality reads (range: 51–132,612 reads per sample; 99.7% samples ≥300 reads; Fig. S1), resulting in 1,112 unique strongylid Amplicon Sequence Variants (ASVs). Altogether, ITS-2 profiles were analysed for 391 samples and nine strongylid genera were identified (Table S3). Briefly, the genus *Murshidia* infecting the large intestine was the most prevalent, detected in nearly all samples across all groups. Stomach parasites of the genera *Paralibyostrongylus* and *Hyostrongylus* were also highly prevalent in both habituated and unhabituated groups. *Oesophagostomum* was more prevalent in habituated gorillas (see details below), and the prevalence increased between 2018 and 2021, with *O. stephanostomum* much more common than *O. bifurcum* (Table S3). *Necator* was only detected in habituated individuals in 2018. Small intestine-dwelling genera *Trichostrongylus* and *Impalaia* were consistently lower in prevalence, and the genera *Nematodirus* and *Cooperia* were detected at very low prevalence in 2018 only.

Visual inspection of strongylid community composition in relation to chronic wasting case counts (Fig. 2) suggested temporal shifts between 2018 and 2021. Groups with higher chronic wasting case counts in 2018 showed increased relative abundances of *Oesophagostomum*, which became more pronounced in 2021 across several groups. The more severely affected groups exhibited distinct strongylid profiles characterized by higher predominance of *Oesophagostomum*, in some cases the genus *Paralibyostrongylus*, and near-absence of the genus *Trichostrongylus*. When paired with EPGS data to provide absolute abundance (Fig. S2), there is a clear shift in 2021 with groups with higher chronic wasting case counts experiencing higher overall strongyle egg counts, usually among only a few individuals in the group with counts surpassing 6,000 EPGS. There was also an individual in Bitukura (a group with zero chronic wasting cases in 2021) with a high egg count but dominated by the genus *Murshidia* and lacking *Oesophagostomum*.

3.1.1. Predictors of strongylid alpha diversity in habituated groups

GLMMs for haplotype richness and LMMs for Shannon and Simpson diversity indices revealed consistent patterns (Table S4). Strongylid diversity was significantly influenced by sampling year (with a notable increase in 2021, all metrics $p < 0.001$), forest position (groups located at forest edge exhibited increased diversity: $p_{richness} = 0.001$, $p_{Shannon} = 0.01$, $p_{Simpson} = 0.04$), and strongylid egg counts (EPGS), which were positively associated with diversity ($p_{richness} = 0.044$, $p_{Shannon} = 0.01$, $p_{Simpson} = 0.02$) (Fig. 3A, B; Fig. S3). Group size, tapeworm burden and chronic wasting case counts were not significant predictors of strongylid diversity (all $p > 0.28$).

3.1.2. Strongylid beta diversity in relation to chronic wasting prevalence category

Principal component analysis of Aitchison distances (PCA) revealed distinct clustering patterns in strongylid communities by chronic wasting prevalence categories (Fig. 3C). The first two principal components explained 49.2% and 17.5% of the total variation. Groups with high chronic wasting prevalence showed distinct community compositions compared to those with low prevalence, with *Oesophagostomum* emerging as the key taxon driving community differences in moderate and high chronic wasting prevalence groups. PERMANOVA with blocked permutations confirmed a strong and significant effect of chronic wasting prevalence category ($R^2 = 0.143$, $F_{2,257} = 23.60$, $p = 0.001$) on strongylid community composition. This test additionally suggested significant effects of sampling year ($R^2 = 0.022$, $F_{1,257} = 7.34$, $p = 0.002$). Forest position and group size were not significant ($p > 0.99$ for both) (Fig. S4). A sensitivity analysis restricted to the 10 groups sampled in both years ($N = 187$ samples) confirmed robustness of these findings, with chronic wasting prevalence category remaining strongly significant ($R^2 = 0.152$, $F_{2,181} = 17.90$, $p = 0.003$), demonstrating that results were not driven by confounding between year and group composition. Pairwise PERMANOVA tests revealed significant differentiation between low- and high-prevalence categories ($R^2 = 33.7\%$, $p = 0.001$) and between low- and moderate-prevalence categories ($R^2 = 18.1\%$, $p = 0.043$), but not between moderate- and high-prevalence categories ($R^2 = 17.0\%$, $p = 0.469$). Centroids in ordination space were separated by Euclidean distances of 0.29 (low vs. moderate), 0.49

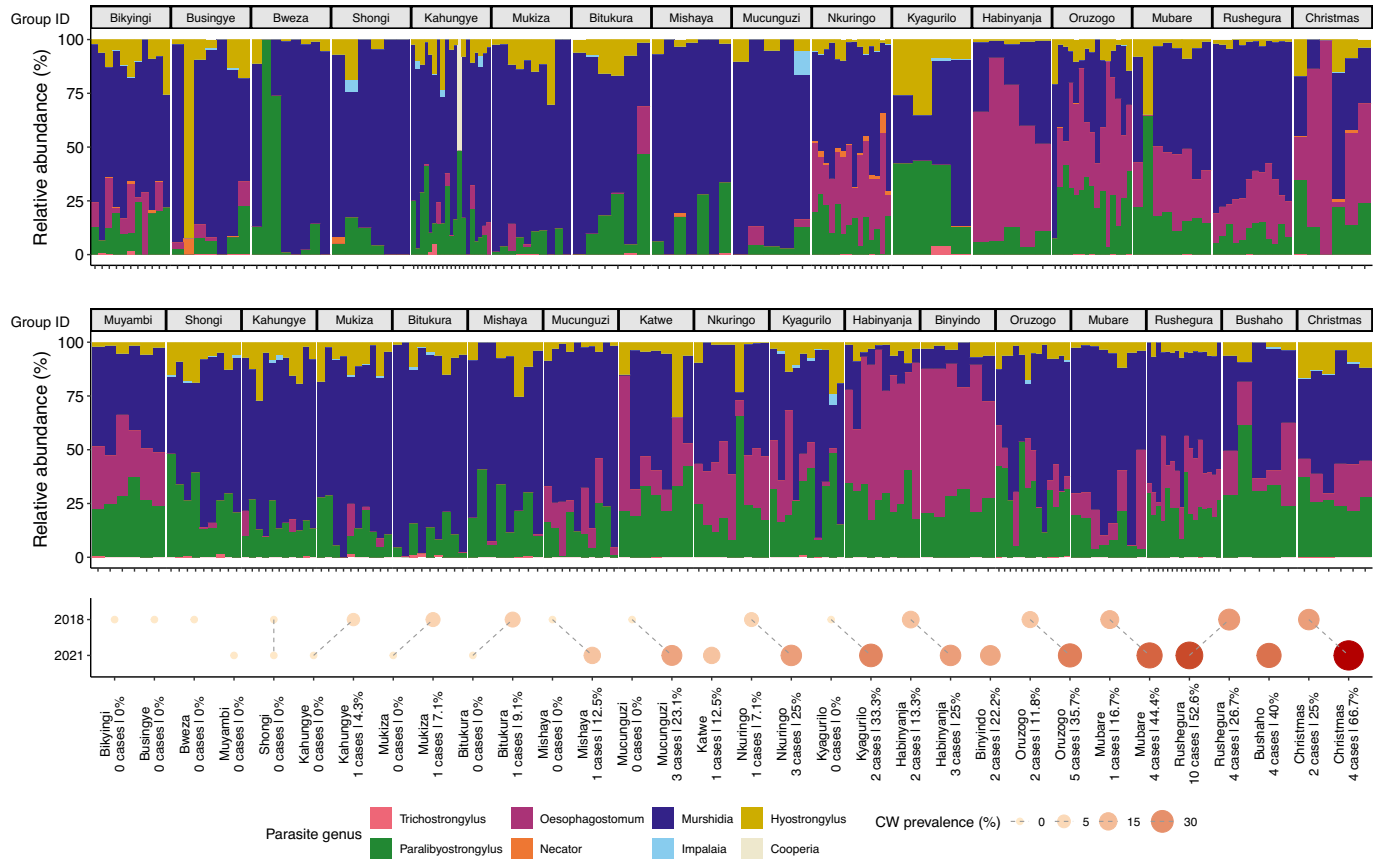


Fig. 2. Relative abundance of strongylid nematode genera in habituated mountain gorilla groups from Bwindi Impenetrable National Park in 2018 (top panel) and 2021 (bottom panel). Each column represents the genus composition for an individual sample, grouped by gorilla group ordered according to the chronic wasting case count which are visualised in the bottom panel, including the between-year change in case count and prevalence.

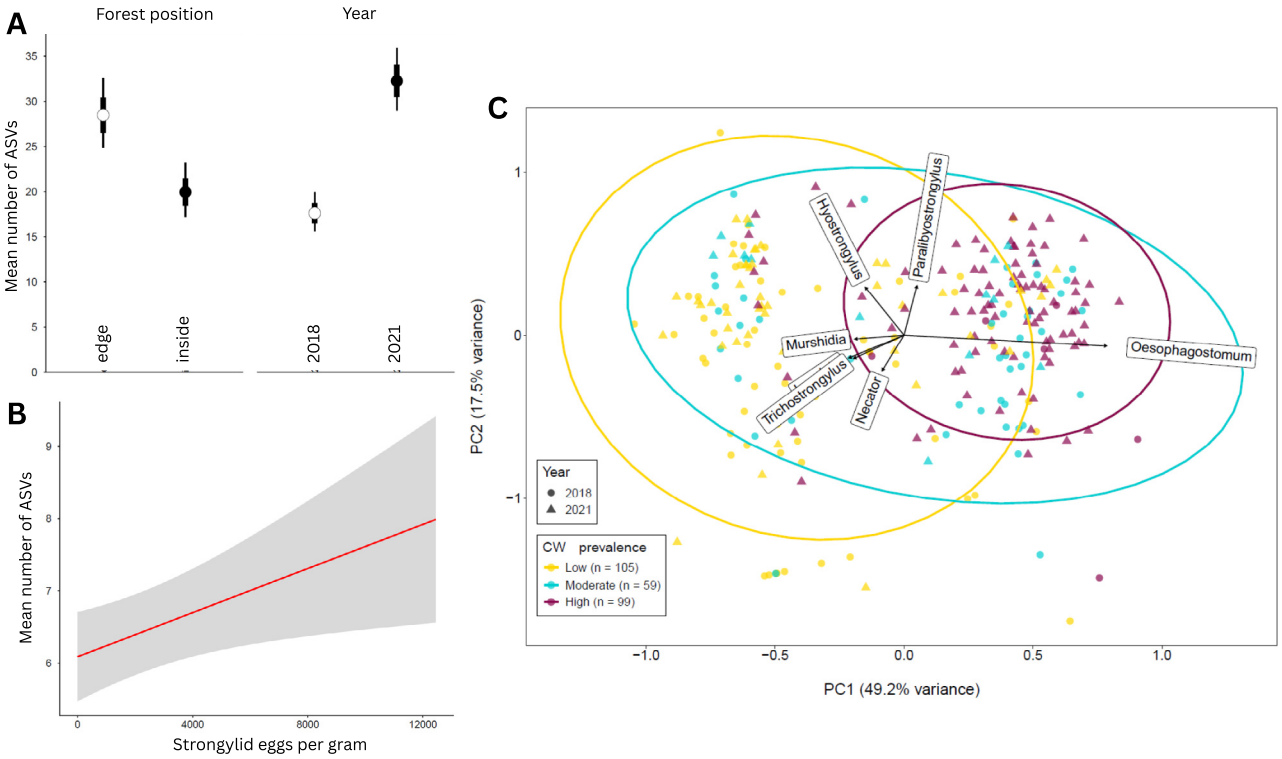


Fig. 3. Effects of (A) gorilla groups' position in forest and sampling year and (B) strongylid faecal egg counts on haplotype richness. Points and lines show predicted means from a generalized linear mixed model with negative binomial distribution. Error bars indicate standard error (thick) and 95% confidence interval (thin). Shaded ribbons show 95% confidence interval. (C) Principal component analysis (PCA) of strongylid communities in habituated mountain gorillas. Points represent individual samples, coloured according to categories of chronic wasting prevalence and shaped by sampling year. Ellipses represent the 95% confidence regions for sample distribution within each chronic wasting prevalence category. Vector arrows show the contribution of different strongylid genera to community variation.

(low vs. high), and 0.20 (moderate vs. high) PC1/PC2 units, with corresponding Cohen's d effect sizes of 0.96, 1.60, and 0.64.

3.1.3. Strongylid taxa associated with chronic wasting occurrence in habituated groups

ANCOM-BC2 identified several strongylid genera with significant differential abundance in relation to chronic wasting prevalence categories (Table S5). Comparisons focused on moderate vs. low and high vs. low groups (low as reference category). *Oesophagostomum* significantly increased relative to the low-prevalence reference in both the moderate (log-fold change [LFC] = 1.43, $q < 0.001$) and high (LFC = 1.32, $q < 0.001$) comparisons. *Trichostrongylus* showed a significant decrease in both moderate (LFC = –0.92, $q < 0.001$) and high (LFC = –1.42, $q < 0.001$) groups, suggesting this genus may be negatively associated with increased chronic wasting prevalence. The genus *Impalaia* showed a modest decrease in moderate compared to low groups (LFC = –0.60, $q = 0.039$) but no significant difference in high vs. low groups. The genus *Murshidia* showed only modest decrease in high vs. low groups (LFC = –0.45, $q = 0.020$) and the genera *Paralibyostrongylus* and *Hyostrongylus* showed no significant differences. Of the significant taxa, genera *Oesophagostomum* (LFC = 1.32–1.43) and *Trichostrongylus* (LFC = –1.42) came closest to the biological significance threshold of $|LFC| \geq 1.5$, while all other significant genera showed substantially smaller fold-changes. The genus-specific GLMM for *Oesophagostomum* confirmed its strong positive association with chronic wasting prevalence category ($\chi^2 = 19.02$, df = 2, $p < 0.001$), with estimated marginal mean absolute abundances of 25.4 (95% CI: 7.7–83.4) EPGS units in low-prevalence groups, 295.6 (95% CI: 84.4–1036.0) in moderate, and 366.6 (95% CI: 101.4–1325.8) in high groups. Post-hoc pairwise contrasts confirmed significant differences between low and moderate groups (ratio = 0.086, $z = -3.66$, $p = 0.001$) and between low and high groups (ratio = 0.069, $z = -3.69$, $p = 0.001$), but not between moderate and high groups (ratio = 0.806, $z = -0.29$, $p = 0.776$).

3.2. Habituation affected strongylid haplotype richness but not community evenness or composition

Comparative visual inspection of strongylid community composition between habituated and unhabituated gorilla groups revealed distinct patterns (Fig. 4, Fig. S5). Unhabituated gorillas were dominated by the genus *Murshidia*, particularly in groups ranging in the southeastern regions of the park. *Oesophagostomum* appeared more prevalent at lower altitudes associated with higher temperature and rainfall (Fig. S6), a pattern observed in both habituated and unhabituated gorillas.

3.2.1. Predictors of strongylid alpha diversity: habituated vs. unhabituated gorillas

GLMMs and LMMs revealed a dissociation between haplotype richness and diversity evenness metrics (Table S3; $N = 238$). Haplotype richness was significantly lower in habituated compared to unhabituated gorillas ($z = -3.46$, $p < 0.001$), indicating reduced ASV-level diversity under habituation. Neither Simpson diversity ($z = -0.33$, $p = 0.74$) nor exponentially transformed Shannon diversity ($z = -0.062$, $p = 0.950$) differed significantly between habituation categories. This pattern suggests that habituation is associated with a reduction in the number of detected strongylid haplotypes, but not with changes in the relative evenness of the community (Fig. 3A, B).

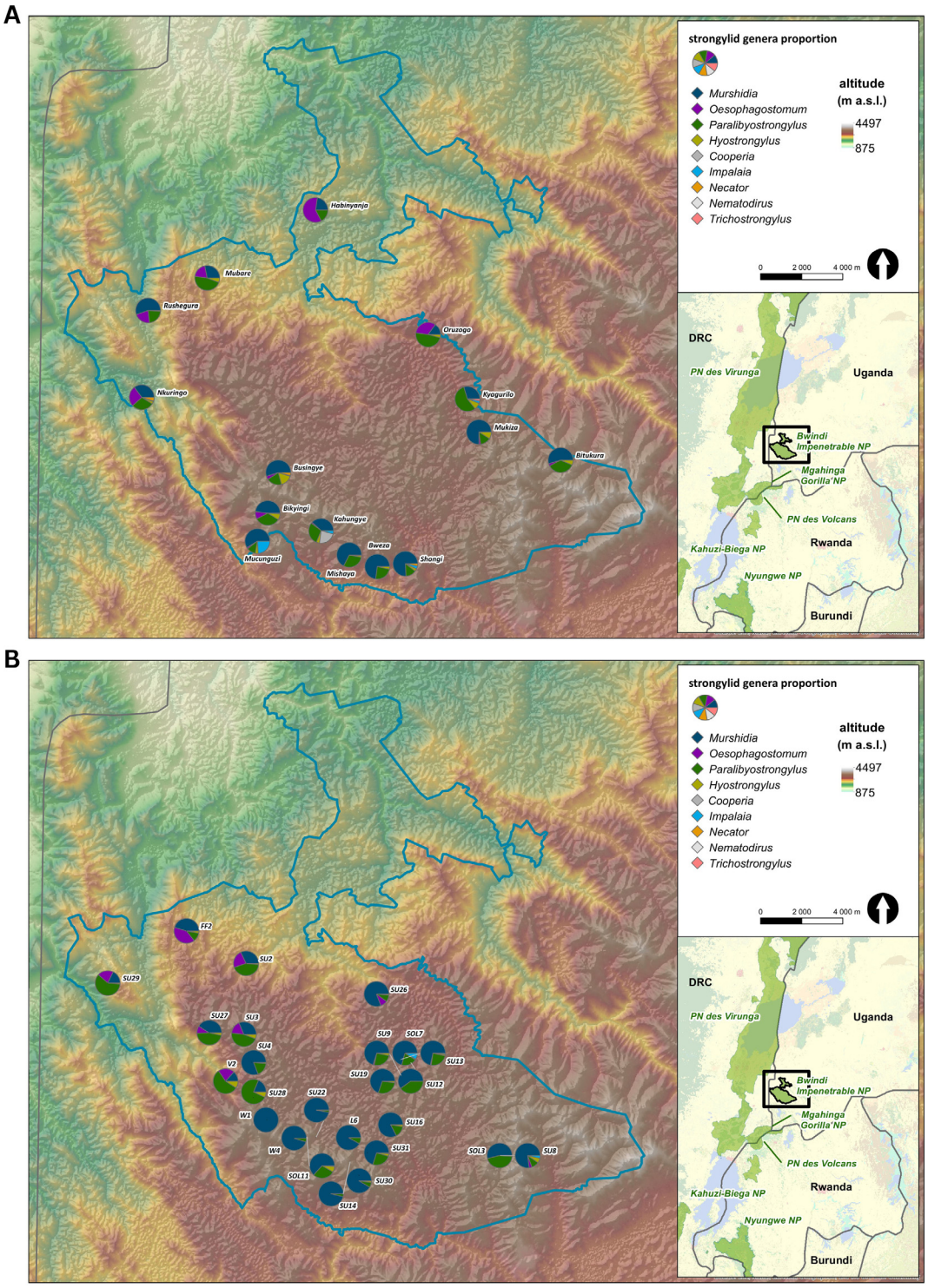


Fig. 4. Map of Bwindi Impenetrable National Park showing the proportional composition of strongylid nematode genera detected in gorilla faecal samples from (A) habituated gorilla groups, and (B) unhabituated gorilla social groups. Codes correspond to identifiers used in the 2018–2019 population census (Hickey et al. 2019). Pie charts indicate the relative abundance of each strongylid genus per group.

3.2.2. Strongylid beta diversity: habituated vs. unhabituated gorillas

Aitchison PCA of CLR-transformed genus-level relative abundances revealed partial separation of strongylid communities by habituation status (Fig. 5), with the first two principal components explaining 47.8% and 19.6% of total variation, respectively. As habituation status is a group-level attribute perfectly nested within group membership, formal PERMANOVA testing of this effect was not possible under the blocked permutation design (see Methods). Nonetheless, habituated gorillas showed greater multivariate dispersion than unhabituated individuals (mean

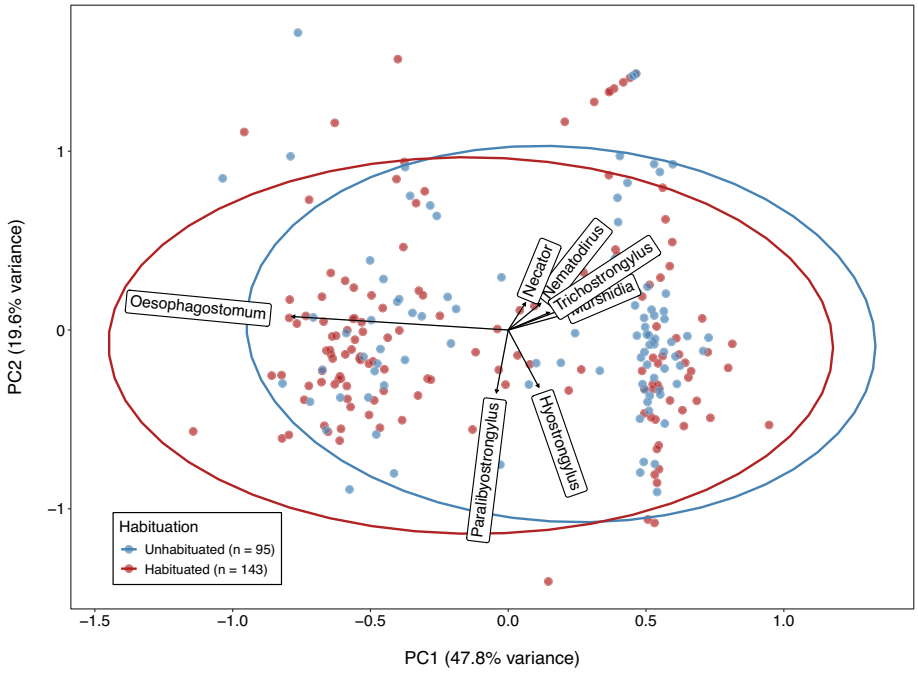


Fig. 5. Principal component analysis of Aitchison distances (PCA) of strongylid communities in mountain gorillas. Points represent individual samples, coloured according to habituation status (habituated and unhabituated groups). Ellipses represent the 95% confidence regions for sample distribution within each category. Arrows show the contribution of different strongylid genera to community variation.

distances to centroid: 4.74 vs. 4.01), suggesting more variable strongylid communities under habituation, though within-group non-independence limits formal inference.

3.2.3. Strongylid taxa associated with habituation

ANCOM-BC2 identified only the genus *Murshidia* with significant differential abundance between habituation categories (Table S6). It showed significantly lower abundances in habituated compared to unhabituated gorillas (LFC = −0.60, $q = 0.01$), though it fell well below the biological significance threshold of $|LFC| \geq 1.5$. All remaining genera showed no significant differences.

3.3. Bacterial community composition was not associated with chronic wasting prevalence

Bacterial 16S rRNA data were obtained for samples collected in 2021 only ($N = 151$; low chronic wasting prevalence: $n = 45$, moderate: $n = 14$, high: $n = 92$), generating 3,401,352 high-quality reads (range: 8,808–31,436 reads per sample), identifying 7,197 bacterial ASVs. The habituated gorilla gut bacterial microbiome is dominated by several ubiquitous orders (Table S7). Coriobacteriales, Oscillospirales, Bacteroidales, Lachnospirales, Peptostreptococcales-Tissierellales, Christensenellales and Erysipelotrichales occurred in all samples. In contrast, orders like Enterobacterales (59.60%), Pseudomonadales (30.46%), and Clostridiales (13.92%, but note Clostridia vadinBB60 group and Clostridia UCG-014 with 99.34 and 74.17 prevalences, respectively) showed more sporadic occurrence. Unlike strongylids, visual inspection of bacterial communities at phylum level in relation to chronic wasting did not reveal distinct patterns; profiles appeared relatively consistent with increasing chronic wasting cases (Fig. 6A, for order level see Fig. S7).

3.3.1. Predictors of bacterial alpha diversity in habituated groups

GLMMs for bacterial ASV richness and LMMs for Shannon and inverse Simpson diversity indices revealed mostly consistent predictors across metrics (Table S8, Fig. S8). Forest position was a significant predictor of ASV richness ($z = -2.40$, $p = 0.02$), with groups categorised as exclusively ranging inside the forest showing lower richness (estimated marginal means: edge = 160 ASVs, 95% CI: 152–167; inside = 145 ASVs, 95% CI: 138–153) and Shannon diversity ($z = -2.10$, $p = 0.04$), though not Simpson diversity ($z = -1.59$, $p = 0.11$). Group size was negatively associated with Shannon ($z = -3.50$, $p < 0.001$) and Simpson diversity ($z = -3.60$, $p < 0.001$), but not ASV richness ($z = -1.44$, $p = 0.15$). Strongylid EPGS counts were negatively associated with Shannon diversity ($z = -3.06$, $p = 0.002$) and Simpson diversity ($z = -2.20$, $p = 0.03$), with a marginal trend for richness ($z = -1.77$, $p = 0.08$). Chronic wasting case counts and tapeworm EPGS were not significant predictors of any alpha diversity metric (all $p > 0.16$) (Fig. S8).

3.3.2. Bacterial beta diversity in relation to chronic wasting prevalence

Aitchison PCA of bacterial communities did not reveal distinct clustering patterns associated with chronic wasting prevalence categories (Fig. 6B), with the first two principal components explaining only 20.9% and 16.4% of the total variation, respectively. As with the habituation analysis above, formal PERMANOVA testing of group-level predictors was precluded by the blocked permutation design (see Methods), and results are therefore presented descriptively. Multivariate dispersion did not differ significantly across chronic wasting prevalence categories ($_{F2,148} = 1.12$, $p = 0.33$; mean distances to centroid: low = 4.62, moderate = 4.26, high = 4.84), confirming that the absence of clustering in the PCA reflects genuine similarity in community composition rather than differences in within-group spread.

3.3.3. Bacterial phyla associated with chronic wasting prevalence

ANCOM-BC2 identified five phyla with significantly increased relative abundance in high compared to low chronic wasting prevalence groups: Desulfobacterota (LFC = 0.91, $q < 0.001$),

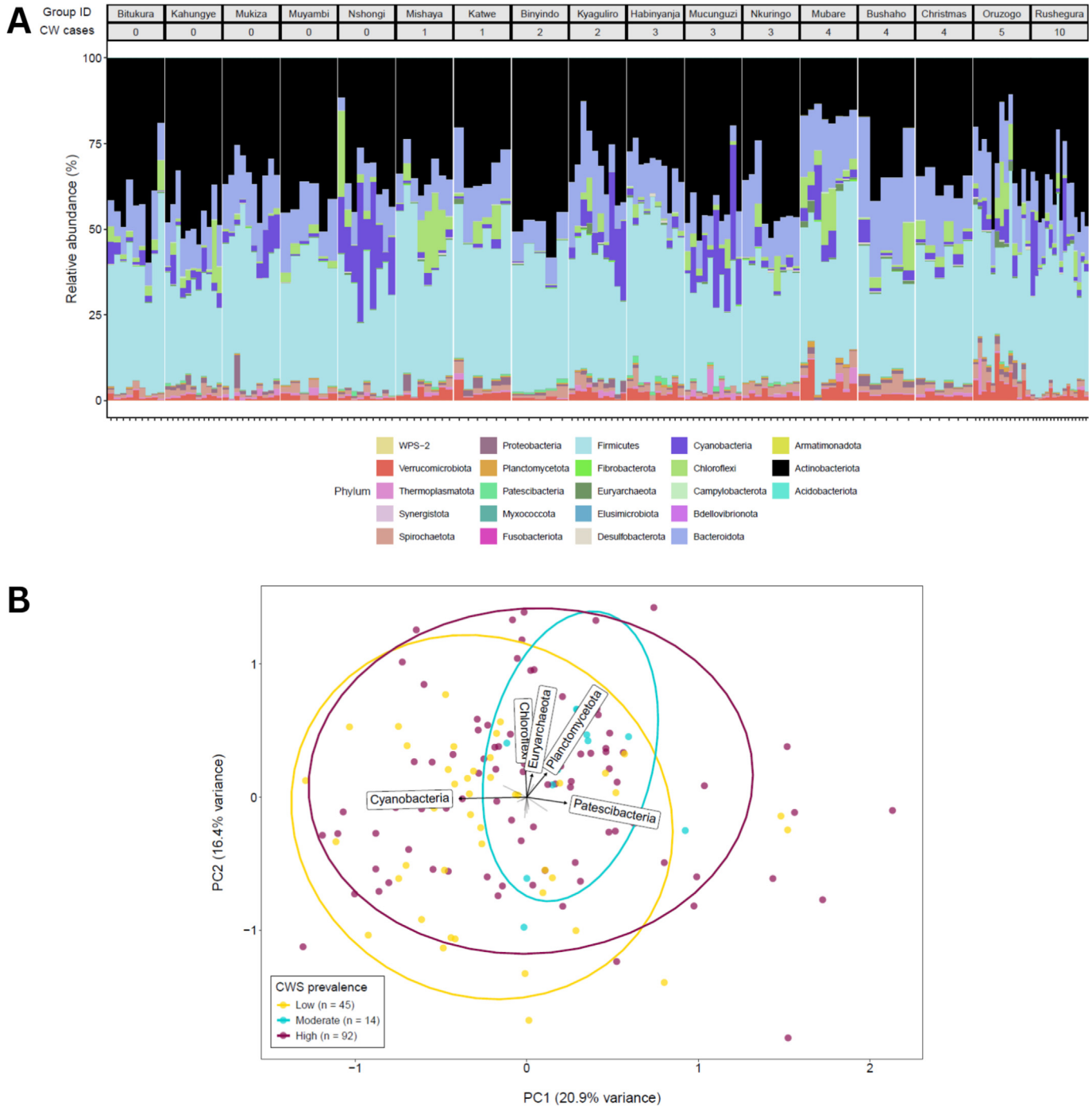


Fig. 6. (A) Relative abundance of bacteria at phylum level in habituated mountain gorilla groups from Bwindi Impenetrable National Park in 2021. Each column represents the composition of 16S rRNA gene ASVs for an individual sample, grouped by gorilla group. Gorilla groups are ordered along the x-axis according to chronic wasting case count. The y-axis shows the relative abundance of bacteria phylum taxa as a percentage of the total community composition within each sample. (B) Principal component analysis of Aitchison distances (PCA) of bacterial communities in habituated mountain gorilla faecal samples. Points represent individual samples, coloured by chronic wasting prevalence categories. Ellipses represent the 95% confidence regions for sample distribution within each chronic wasting occurrence category. Arrows show the contribution of different bacterial phyla to community variation.

Patescibacteria (LFC = 0.85, $q < 0.001$), Euryarchaeota (LFC = 0.67, $q = 0.001$), Verrucomicrobiota (LFC = 0.65, $q = 0.008$), and Planctomycetota (LFC = 0.53, $q = 0.004$) (Table S9). In the moderate vs. low comparison, only Desulfobacterota reached statistical significance (LFC = 0.99, $q = 0.020$). However, all LFC values fell substantially below the biological significance threshold of $|LFC| \geq 1.5$, suggesting that these differences, while statistically detectable, are of limited biological relevance.

3.4. Strongylid–bacteriome covariation

The Mantel test revealed a small but statistically significant positive correlation between overall strongylid and bacterial community dissimilarity ($r = 0.13$, $p = 0.002$, $N = 151$ samples), indicating that individuals with more similar strongylid communities also tend to harbour more similar bacterial communities, though the effect is weak.

At the genus level, pairwise Spearman correlations between CLR-transformed strongylid genera (≥10% prevalence) and bacterial genera (≥10% prevalence), with Benjamini–Hochberg correction across all tests, identified 24 significant pairs ($q < 0.05$; Table S10). Effect sizes were modest ($|\rho| = 0.25–0.37$). Three strongylid genera drove most of the significant associations: *Murshidia*, *Oesophagostomum*, and *Impalaia*; *Trichostrongylus* contributed one association. The genus *Murshidia* showed the greatest number of significant correlations ($n = 13$), spanning multiple bacterial phyla. *Oesophagostomum* yielded eight and the genus *Impalaia* three significant associations.

4. Discussion

Our analyses revealed a marked restructuring of strongylid communities in mountain gorillas in relation to chronic wasting occurrence, with *Oesophagostomum* emerging as the principal taxon associated with these shifts. Groups with higher wasting prevalence were characterised by increased prominence of *Oesophagostomum* and a reduction in certain trichostrongylid taxa. These patterns were consistent across sampling years and exceeded the effects attributable to forest position or habituation status. In contrast, bacterial community composition exhibited comparatively limited variation with respect to wasting. Collectively, these findings indicate that chronic wasting is associated with specific alterations in strongylid assemblages rather than broad-scale microbial disruption and identify particular helminth taxa as priorities for further investigation.

Before interpreting these patterns further, it is important to acknowledge a methodological consideration inherent to metabarcoding approaches based on whole faecal extracts. The exact number of parasite eggs contributing to the extracted DNA could not be determined, as sequencing was performed on homogenized faecal material rather than individually recovered eggs. As a result, read proportions represent relative amplicon abundance following PCR amplification and library normalization rather than direct measures of egg counts. In samples with low egg counts, stochastic effects during DNA extraction and amplification may increase variability and reduce the detectability of rare taxa. However, the consistent recovery of dominant genera across samples, including those with lower EPGS, suggests that sequencing depth and analytical resolution were sufficient to capture the principal components of the strongylid community, consistent with previous applications of this approach. Importantly, the taxa most strongly associated with chronic wasting were detected at substantial relative abundance and were not restricted to low-intensity samples, reducing the likelihood that the observed patterns are artefacts of low egg input.

Across the study period, strongylid communities were consistently dominated by the genera *Murshidia, Paralibyostrongylus*, and *Hyostrongylus.* Notably, *Oesophagostomum* prevalence increased over time with the rise in chronic wasting cases (from 59.44% in 2018 to 73.20% in 2021). Strongylid alpha diversity also increased significantly from 2018 to 2021, with higher strongylid EPGS contributing to greater ASV diversity, as may be expected. This increased diversity may be driven by movement among social groups, genetic mutations, or introduction of novel haplotypes from other hosts. Conversely, strongylid haplotype richness was reduced in groups ranging further inside the forest, suggesting that edge disturbance characterised by increased human activity, livestock presence, habitat fragmentation, and altered microclimatic conditions may enhance parasite diversity. This contrasts with findings that link higher parasite richness to conserved habitats (Solórzano-García et al., 2023) but is consistent with broader principles showing that disturbance can alter host–parasite dynamics (Bitters et al., 2022). Habitat fragmentation and increasing human–wildlife contact can also facilitate parasite spillover by altering host community structure and disrupting long-standing host–parasite associations (Morand and Lajaunie, 2021).

Across analyses, chronic wasting prevalence was the strongest predictor of strongylid community structure, while sampling year and forest position were important mainly for alpha diversity, with forest position not significant in the multivariate community-composition model. High-chronic wasting groups harboured distinct parasite communities, with *Oesophagostomum* spp. emerging as the key taxon differentiating these groups from those with lower disease occurrence. ANCOM corroborated this pattern. Conversely, the genus *Trichostrongylus* decreased significantly in groups with moderate and high occurrence of chronic wasting compared to low occurrence groups. Both genera inhabit the intestine, suggesting that *Trichostrongylus* may be outcompeted by *Oesophagostomum* in individuals with high parasite burden. The abundance of two additional trichostrongylids, *Paralibyostrongylus* and *Impalaia*, differed significantly in relation to chronic wasting occurrence. While the genus *Impalaia* showed only a modest and inconsistent trend, *Paralibyostrongylus* did not differ significantly among chronic wasting categories, suggesting that neither taxon is likely to be a major driver of chronic wasting.

Oesophagostomum (commonly known as nodular worms) are well-characterised gastrointestinal nematodes with a long history of documented pathology in humans, livestock, and non-human primates (Stewart and Gasbarre, 1989), with disease expression strongly dependent on infection intensity, host immunity, and ecological context. *Oesophagostomum* infections have been repeatedly documented in African great apes, including gorillas, but their distribution and epidemiological importance appear to vary between gorilla populations. In western lowland gorillas (*Gorilla gorilla gorilla*), *Oesophagostomum* are commonly detected and are typically part of the background strongylid community, although faecal egg counts are generally low in western lowland gorillas compared with those observed in Bwindi mountain gorillas (Makouloutou et al., 2014; Sirima et al., 2021; Ilík et al., 2023). In contrast, these strongylids appear to be rare or absent in mountain gorillas of the Virunga Massif, where parasitological surveys have not reported substantial *Oesophagostomum* infections (Mason et al., 2025; Pafčo et al., 2026). This marked difference between neighbouring mountain gorilla populations suggests that ecological or transmission-network factors may influence the establishment and persistence of this parasite. One possible explanation is the absence of chimpanzees in the Virunga ecosystem. Because chimpanzees are known hosts of *Oesophagostomum* and often carry high infection intensities, their presence in Bwindi may contribute to maintaining environmental transmission cycles that also affect sympatric gorillas. The absence of chimpanzees in Virunga therefore raises the hypothesis that cross-species transmission or shared environmental contamination could be important for sustaining *Oesophagostomum* circulation in Bwindi gorillas.

Oesophagostomum infections have caused fatal outcomes in captive primates and are increasingly recognized as significant pathogens in wild populations. In Tanzania, nodular worms have been associated with mortality in baboons and chimpanzees (Terio et al., 2018), while in Taï National Park (Côte d'Ivoire), *O. stephanostomum* has caused parasitic granulomas and severe intestinal pathology in wild chimpanzees (Jaffe et al., 2024). Similarly, in the Mahale Mountains (Tanzania), infections led to large nodular lesions in the chimpanzees' intestinal wall, causing substantial pathology and prompting self-medicative plant use during periods of peak infection (Huffman et al., 1997). A study on post-mortem pathological lesions in the digestive tracts of 60 free-ranging mountain gorillas identified nodular worm infections, but the associated lesions were not as prevalent as expected based on known

prevalence of this parasite in the population and the overall impact of these lesions on health were unclear (Muhangi et al., 2021). Notably, the examined animals died between 1985 and 2007, and our results suggest that infection dynamics, including dominance of *Oesophagostomum* in chronic wasting cases may have shifted since then.

Our findings build on this established framework by demonstrating that, under current ecological conditions in Bwindi, *Oesophagostomum* may be contributing to a clinically observable chronic wasting in a wild great ape population. The concurrent increase in *Oesophagostomum* prevalence and abundance alongside rising chronic wasting cases suggests a substantial impact on mountain gorilla health. Confined ranges may sustain constant exposure to infective larvae. In livestock, repeated *Oesophagostomum* infections cause calcified nodules and chronic pathology, unlike typically acute trichostrongylid infections (Deplazes et al., 2016). Experimental studies in non-human primates show that *Oesophagostomum* L4 larvae exit nodules rapidly in immunologically naive individuals, whereas immune animals develop caseous nodules (Polderman et al., 2010; Ruch, 1959) and more numerous parasitic granulomas were also recorded in older chimpanzees in Gombe (Terio et al., 2018). These findings suggest that reinfections may be significant for great ape health. *Murshidia* nematodes, like *Oesophagostomum*, also reside in the large intestine and form nodules (unpublished data, on file). In Virunga, gorilla groups with high absolute abundance of the genus *Murshidia* showed no clinical signs (Pafčo et al., 2026). However, co-infection with the two nodule-forming species in Bwindi could potentially exacerbate the clinical outcomes, although at present this remains speculative.

While our results demonstrate a strong association between *Oesophagostomum* and chronic wasting, they cannot definitively establish directionality. It is possible that chronic wasting reflects an underlying impairment of host condition, or immune function or genetic predisposition, which could in turn permit higher *Oesophagostomum* establishment, survival, or fecundity. In such a scenario, elevated egg counts or relative sequence abundances might reflect reduced immune-mediated suppression of worm reproduction (Maizels and Yazdanbakhsh, 2003) rather than a primary causal role of the parasite in initiating disease. Importantly, faecal egg counts and metabarcoding data primarily reflect patent infections and egg output, not total worm burden. *Oesophagostomum* larvae encyst within the intestinal mucosa, where they can induce nodular pathology (Polderman and Blotkamp, 1995). These tissue-dwelling stages (as well as adult males) are not detected by egg detection-based methods, and no non-invasive marker currently exists to quantify true larval burden in live animals. Therefore, it remains possible that pathology is driven in part by larval stages that are underrepresented in faecal-based estimates. Longitudinal clinical monitoring combined with improved markers of infection intensity would be required to determine causality and associated sequalae.

Anthelmintic treatment (eprinomectin) leads to short-term clinical improvement of treated animals (Gorilla Doctors, data on file), but larvae encapsulated in granulomas or abscess-like nodules within the intestinal wall (Terio et al., 2018) may be shielded from the drug's effect, especially in case of parenteral administration (Craig, 2009). At >14 days post single dose treatment with eprinomectin we note a significant decrease or elimination of other strongyle species but not *Oesophagostomum* or *Murshidia* (Fig. S9). This raises concern over the potential for single dose treatment regimens to contribute to anthelminthic resistance against the avermectin drug class, the only available injectable treatment option for strongyle infections. Treatment of wild gorillas is notably challenging, particularly when repeated doses of anthelmintic medication are needed: gorillas quickly learn to avoid being darted which can sometimes result in only one dose effectively being delivered. Anthelmintic resistance is well-documented (Fissiha and Kinde, 2021) and may be exacerbated at forest edges, where habituated groups are more frequently treated. Environmental accumulation also poses risks: BINP's high rainfall can disperse residues through the water table, with potential toxicity for aquatic organisms (Mooney et al., 2021), while routine livestock treatments and human anthelmintic use around the park may further drive resistance. Chimpanzees and bonobos self-medicate against these parasites by leaf swallowing (Fruth et al., 2014; Dupain et al., 2002), a mechanical mechanism to expel adult worms from the intestines triggering larvae to leave nodules (Huffman et al., 1996). Surgical removal has been effective (Ngoubangoye et al., 2021) but this is exceptionally difficult, if not impossible to conduct on a wild primate, especially with ethical considerations.

Oesophagostomum species found in non-human primates are of zoonotic concern, although most documented human infections involve *O. bifurcum*, which appears to be rare in Bwindi mountain gorillas (Table S3, Fig. S10). In contrast, nearly all *Oesophagostomum* haplotypes detected in this study were assigned to *O. stephanostomum*, a species primarily associated with African great apes but occasionally reported in humans (Ghai et al., 2014). In Uganda, three *Oesophagostomum* taxa, probably all species, have been described in primates (*O. stephanostomum, O. bifurcum*, and an undetermined *Oesophagostomum* sp.), with molecular studies demonstrating genetic overlap between human and non-human primate infections (Ghai et al., 2014; Cibot et al., 2015). Notably, a widely distributed *O. stephanostomum* haplotype referred to as type I has previously been identified across humans and multiple non-human primate species in Uganda and Cameroon (Cibot et al., 2015; Ilík et al., 2023), and this same haplotype was detected in the present study. Occurrence of a zoonotic haplotype in the mountain gorillas highlights the importance of considering parasite genetic diversity and host–parasite co-evolution within ecosystem health and One Health frameworks linking conservation and public health (Adisasmito et al., 2022).

Habituation to humans, particularly through frequent tourist visits, may subtly but significantly affect gorilla stress physiology. In Bwindi, self-directed behaviours, indicative of psychological stress, increased with tourist proximity (Costa et al., 2024). Chronic stress can impair immunity, raising concerns about the long-term health impacts of ecotourism. Although habituation was associated with lower strongylid haplotype richness, its effects on overall community composition were limited and could not be tested formally under the blocked permutation design. These findings align with previous studies in Virunga gorillas, where habituation effects on strongylid intensities were minor (Petrželková et al., 2022), and lowland gorillas, where habituation did not increase risk of protist or helminth infections (Pafčo et al., 2017). Moreover, habituated or semi-habituated gorillas did not exhibit greater strongylid overlap with humans than unhabituated groups (Pafčo et al., 2019).

Unhabituated gorillas also carried *Oesophagostomum*, but only where nearby habituated groups had high *Oesophagostomum* abundance, suggesting that environmental contamination and accumulation may play larger roles than habituation alone. Heavily affected habituated chronic wasting groups exhibiting high relative abundances of *Oesophagostomum* were primarily located in the lower altitudes, in the northwestern part of Bwindi. This spatial pattern may parallel findings from the Virunga Massif, where *Hyostrongylus*-dominated communities were shaped by elevation, rainfall, and vegetation (Pafčo et al., 2026). We cautiously propose that similar environmental drivers may be contributing to the emergence of pathogenic *Oesophagostomum*-abundant communities in Bwindi, particularly in lowland regions. Climate change may compound risks; rising temperatures in Bwindi have been linked to increased use of water sources by gorillas (Wright et al., 2022), potentially creating additional opportunities for

pathogen accumulation and transmission. Future work should integrate high-resolution GIS on vegetation, climate, and elevation to test ecological predisposition to chronic wasting and assess shifts in plant-based self-medication with potential anthelmintic properties.

Interestingly, *Murshidia* was the main genus to be found to have some significant interactions with the bacteria genera. This likely reflects the fact that *Murshidia* was the most prevalent strongylid nematode infecting the studied gorillas. However, bacterial 16S metabarcoding alone is not sufficient to precisely evaluate the functional role of the associated bacteria. This is because the taxonomic resolution of 16S rRNA metabarcoding is often limited to the genus level, and many bacterial genera may include both beneficial and pathogenic species that cannot be further distinguished with this approach. Despite this limitation, we did not observe a consistent pattern in the correlations between strongylid nematodes and bacterial genera. Both potentially beneficial bacteria, commonly associated with carbohydrate metabolism and fibre-rich diets, and taxa that have been linked to dysbiosis or disease in humans were detected among the interacting genera.

Several positively associated taxa, including *Parabacteroides*, *Intestinimonas*, *Ruminococcaceae* UCG-005, and the *Eubacterium hallii* group, are recognised fibre-degrading and short-chain fatty acid (SCFA)–producing commensals that contribute to butyrate production, metabolic regulation, and maintenance of intestinal barrier integrity in mammalian systems (Engels et al., 2016; Liu et al., 2025; Oh et al., 2022; Rampanelli et al., 2025). Their involvement suggests potential links between helminth presence and microbial pathways associated with fermentation and gut homeostasis. At the same time, *Murshidia* and *Oesophagostomum* were also associated with taxa that, in other systems, are linked to inflammatory or dysbiotic states. These included *Erysipelatoclostridium*, *Mogibacterium*, *Solobacterium*, and *Coriobacteriaceae* UCG-003, which have been associated with intestinal inflammation, metabolic disturbance, or opportunistic behaviour under disrupted gut conditions (Gao et al., 2022; Ma et al., 2024; Yu et al., 2024; Zakerska-Banaszak et al., 2025).

Positive correlations with *Prevotellaceae* UCG-001 and *Treponema* are notable, as these genera are often enriched in high-fibre, non-industrialized gut microbiomes and are involved in complex carbohydrate degradation (Angelakis et al., 2019; Flint et al., 2008), yet some *Prevotella* lineages have also been linked to inflammatory phenotypes (Ley, 2016). Associations with *Acinetobacter* and *Campylobacter*, genera that include opportunistic or pathogenic species (Wong et al., 2017; Wu et al., 2022), further suggest that higher strongylid burdens may coincide with increased representation of potentially inflammatory or environmentally derived taxa. Importantly, both positive and negative correlations were detected across taxa with contrasting ecological roles.

At a broader scale, the most common taxa identified, Firmicutes, Actinobacteria and Bacteroidota, aligned with previous studies of the Bwindi mountain gorilla microbiome (Gomez et al., 2016; Rühlemann et al., 2024; Sharma et al., 2022). Differential abundance analysis (ANCOM-BC2) identified five phyla; Desulfobacterota, Patescibacteria, Euryarchaeota, Verrucomicrobiota, and Planctomycetota, as significantly enriched in groups with high chronic wasting prevalence compared to low prevalence groups, with Desulfobacterota also elevated in the moderate versus low comparison. The phylum Desulfobacterota includes sulphate-reducing bacteria that have been associated with inflammatory gut environments and altered mucosal conditions in other systems (Singh et al., 2023). The consistent enrichment of this phylum in higher chronic wasting prevalence groups suggests that chronic wasting is accompanied by targeted taxonomic shifts rather than wholesale microbiome disruption. Despite these taxon-specific differences, overall community structure remained stable. Aitchison PCA did not reveal distinct clustering by chronic wasting category, and multivariate dispersion did not differ significantly among groups. Chronic wasting was therefore not associated with discrete microbiome "types," but with more subtle compositional shifts.

Alpha diversity patterns, however, revealed clearer ecological effects. Strongylid egg counts were negatively associated with bacterial Shannon and Simpson diversity, with a marginal trend toward reduced ASV richness. Higher parasite burdens were therefore linked primarily to reduced community evenness, indicating that a smaller subset of taxa dominates the microbiome under elevated helminth pressure. The number of bacterial taxa remained relatively similar across EPGS levels, yet their relative abundances shifted. Group size also negatively predicted Shannon and Simpson diversity but not richness, suggesting that larger gorilla groups harbour less even microbial communities, potentially reflecting increased microbial transmission or dietary convergence within social units. Forest position predicted ASV richness, with groups located inside the forest exhibiting lower richness than edge-ranging groups, which may encounter a broader environmental microbial pool, including taxa associated with anthropogenic interfaces. Taken together, these findings suggest that gut microbial communities in mountain gorillas are shaped by an interplay of parasite burden, chronic wasting status, social structure, and ecological context. Rather than reflecting a simple dysbiotic collapse, the observed patterns are consistent with gradual ecological rebalancing of fermentation-associated, inflammation-linked, and environmentally derived taxa under increasing parasite pressure and host physiological stress.

In conclusion, our study reveals a strong link between the rising incidence of chronic wasting in Bwindi mountain gorillas and notable shifts in strongylid nematode communities, particularly the increasing abundance of pathogenic *Oesophagostomum*. These parasites may now pose a significant emerging health threat to this endangered population. By leveraging metabarcoding, we demonstrate the value of non-invasive tools for gut ecosystem–health inference. Moving forward, integrative approaches combining eukaryotic and prokaryotic microbiomes, metabolites, and environmental data are essential. A One Health framework spanning humans, livestock, and sympatric wildlife will clarify cross-species risks and guide interventions. Ultimately, safeguarding mountain gorillas requires addressing ecological drivers, anthropogenic pressures, and zoonotic risks in parallel to ensure the long-term survival of this endangered species.

Data accessibility statement

The data that support the findings of this study are available in figshare at https://doi.org/10.6084/m9.figshare.30265147. Raw sequencing data are archived in the European Nucleotide Archive under the accession number of the whole project PRJEB111602 (available at: https://www.ebi.ac.uk/ena/browser/view/PRJEB111602). Accession number for each sample is available in Supplementary table 11.

CRediT authorship contribution statement

Kelly Marie Sambucci: Writing – review & editing, Writing – original draft, Project administration, Investigation, Formal analysis, Data curation. **Peter Samaš:** Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis. **Benard Ssebide:** Writing – review & editing, Resources, Project administration, Data curation, Conceptualization. **Klára Judita Petrželková:** Writing – review & editing, Writing – original draft, Supervision, Methodology, Investigation, Funding acquisition, Formal analysis, Conceptualization.

Ricky Okwir Okello: Writing – review & editing, Resources, Project administration, Methodology, Data curation. **Fred Nizeyimana:** Writing – review & editing, Project administration, Investigation, Data curation. **Nelson Bukamba:** Writing – review & editing, Investigation, Data curation. **Tierra Smiley-Evans:** Writing – review & editing, Supervision, Project administration, Methodology, Investigation, Formal analysis, Data curation. **Kirsten Gilardi:** Writing – review & editing, Writing – original draft, Supervision, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Barbora Pafčo:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Methodology, Investigation, Formal analysis, Conceptualization. **Barbora Červená:** Writing – review & editing, Writing – original draft, Validation, Supervision, Resources, Project administration, Methodology, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Funding

This research was funded by The Czech Science Foundation GACR (22-16475S, 26-23106S), and the Morris Animal Foundation (D21ZO-042). We acknowledge the CF Genomics and the CF Bioinformatics supported by the NCMG research infrastructure (LM2023067 funded by MEYS CR) for their support with obtaining scientific data presented in this paper. Computational resources were provided by the e-INFRA CZ project (ID:90254), supported by the Ministry of Education, Youth and Sports of the Czech Republic.

Acknowledgements

We extend gratitude to the Uganda Wildlife Authority and the Uganda National Council of Science and Technology for their permission and long-term support for conducting this research. All samples were collected noninvasively, adhering to research protocols and regulations at all research sites, while also complying with all legal requirements of Uganda.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijpara.2026.104848.

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