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Protist Infections in Mountain Gorillas across Virunga Massif

MASTER'S THESIS

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Declaration

I hereby declare that I have done this thesis entitled **Protist infections in mountain gorillas across Virunga Massif** independently, all texts in this thesis are original, and all the sources have been quoted and acknowledged by means of complete references and according to Citation rules of the FTA.

In Prague, 15th August 2022

……..
Carine UWAMAHORO

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Abstract

Mountain gorilla (*Gorilla beringei beringei*) populations have grown steadily in recent decades, thanks to intensive conservation efforts in Rwanda, Uganda and the Democratic Republic of Congo. Achieved conservation success coincides with increasing population densities, which may alter parasite epidemiology. Recently, striking geographic differences in helminth infections were revealed in mountain gorillas inhabiting Virunga Massif, with higher egg counts and specific strongylid taxa occurring in high-elevation areas with high occurrence of clinical gastrointestinal symptoms. However, the knowledge about protist diversity and epidemiology in mountain gorilla populations has been still limited. The goal of my study was to assess patterns of microsporidia, *Cryptosporidium* and *Giardia* infections (intestinal protists spread by the fecal-oral route via environmentally resistant cysts) in mountain gorilla across the Virunga Massif. Using molecular diagnostics (PCR and qPCR on various markers) I examined 152 fecal samples from 16 gorilla groups collected non-invasively in 2018. I detected *Enterocytozoon bieneusi* genotype D (14%) and genotype CHN-F1 (less than 1%), *C. muris* (less than 1%) and *Giardia intestinalis* (13%). I found no spatial and sex/age differences in microsporidia, *Cryptosporidium* and *Giardia* infections, but my results pointed to the shifts in studied protist communities in Virunga gorillas compared to the published results from 2007 data. I showed that the most sensitive methods for pathogen detection must be employed and a long-term monitoring of selected pathogens is warranted. My results further aid 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: Nonhuman Primate, Gorilla, Protist, *Cryptosporidium*, *Giardia*, microsporidia, Health, Conservation,

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List of the abbreviations used in the thesis

DFGFI:	Dian Fossey Gorilla Fund International
MGVP:	Mountain Gorilla Veterinary Project
RDB:	Rwanda Development Board
PCR:	Polymerase Chain Reaction
qPCR:	quantitative Polymerase Chain Reaction
NIC:	Normarski Interference Contrast
IUCN:	International Union Conservation of Nature
DRC:	Democratic Republic of Congo
DNA:	Deoxyribonucleic Acid
EVD:	Ebola Virus Disease
rRNA:	Ribosomal Ribonucleic Acid
ITS:	Internal Transcriber Spacer
TPI:	Triosephosphate Isomerase
DIC:	Digital Interference Contrast
OPG:	Oocyst Per Gram
MIF:	Merthiolate Iodine Formaldehyde
VNP:	Volcanoes National Park
MASL:	Meters Above Sea Level
MGNP:	Mgahinga Gorilla National Park
TAE:	Tris-Acetate-EDTA
MAFFT:	Multiple Alignment Fast Fourier Transform
GLMM:	Generalized Linear Mixed Model
GDH:	Glutamate Dehydrogenase
ML:	Maximum Likelihood

1. Introduction and literature review

1.1. Introduction

Mountain gorillas are endemic to the Virunga Massif and Bwindi – Sarambwe ecosystem. Due to their small population size and the different threats mountain gorilla are facing, they are classified as an endangered species. The extreme conservation efforts received by mountain gorilla populations has led to their continuous growth (Gray et al. 2013; Hickey et al. 2019a). However, as the potential for spatial expansion is limited due to dense human communities living nearby, this growth resulted in higher gorilla densities and more groups, with higher home range overlaps among neighboring groups Caillaud et al. (2014), which can likely affect the pathogen epidemiology and their susceptibility to infectious diseases.

Gastrointestinal problems (chronic gastritis) and chronic wasting cases linked to helminth infections have been already observed in the Virunga and Bwindi gorillas respectively (Muhangi et al. 2021) and may pose a threat to these endangered animals. This situation led to joint research efforts of scientists, veterinarians and conservationists, from Mountain Gorilla Veterinary Project (MGVP, aka Gorilla Doctors), Dian Fossey Gorilla Fund International (DFGFI), Rwanda Development Board (RDB), Institute of Vertebrate Biology, Czech Academy of Sciences and University of Veterinary Sciences Brno, with the aim to clarify helminths patterns across mountain gorilla populations (Petrželková et al. 2021, 2022). Up to now, striking geographic differences in strongylid infections (both intensity of infection and community composition) within the Virunga Massif have been revealed, with higher egg counts and specific gastric strongylid taxa occurring mostly in areas with high occurrences of gastric disease (area between Karisimbi and Visoke) (Petrželková et al. 2021, 2022). This highly collaborative research pointed to new challenges emerging as possible "side effects" of the remarkable conservation success of the past few decades and further called for examining other parasite infections in mountain gorillas. Unraveling the patterns of parasite infections in both gorilla populations, evaluating host exposure to infective parasite stages, and

studying susceptibility to infection and its consequences on host health, is an important next step for the survival of small, isolated populations of mountain gorillas.

Only limited work has been done to explore the molecular diversity of potentially pathogenic gastrointestinal protists in mountain gorillas, especially in the Virunga Massif, with exception of the studies by (Sak et al. (2014) and Hogan et al. (2014), showing presence and a diversity of important opportunistic protists (unicellular parasites), namely microsporidia, *Giardia* sp. and *Cryptosporidium* spp. in Virunga gorillas. These protists are transmitted through the fecal-oral route via environmentally resistant stages (Modrý et al. 2018). In this thesis, I focused on exploring microsporidia, *Giardia* sp. and *Cryptosporidium* infections in Virunga population of mountain gorillas.

1.2. Literature Review

1.2.1. Mountain Gorilla

There are two gorilla species, western gorilla (*Gorilla gorilla*) and the eastern gorilla (*Gorilla beringei*). Mountain gorilla (*Gorilla beringei beringei*) is a subspecies of the eastern gorilla. Mountain gorillas are currently classified by the International Union for the Conservation of Nature (IUCN) as Endangered due to various threats faced in their limited habitats, including anthropologic activities, climate change and infectious diseases (Hickey et al. 2020). The mountain gorillas live in the Afromontane Forest that spans to the borders of Rwanda, Uganda and the Democratic Republic of Congo (DRC). They are found in Virunga National Park and Sarambwe Reserve in DRC; Volcanoes National Park in Rwanda; Mgahinga Gorilla National Park and Bwindi Impenetrable National Park in Uganda (Figure 1).

Mountain gorillas live in family groups, which range in size, with an average of 10 family members; however, some can have up to 40 individuals including infants, juveniles, subadults and adults. A group may have several females and males, but the lead belongs to one dominant silverback (Schaller 1963). Mountain gorillas are herbivorous, and 85% of their diet is comprised of leaves, shoots and steams (Watts 1984). However, mountain gorillas can also eat roots, larvae, snails, termites and ants (Watts 1998). Mountain gorillas can weigh between 90 kg to 180 kg for adult individuals, and they are

closely related to humans, sharing 98% of human DNA (Gladys et al. 2004), which can suggest an easy transmission of diseases from gorillas to humans and *vice versa* (Ryan et al. 2014; Thompson & Ash 2016).

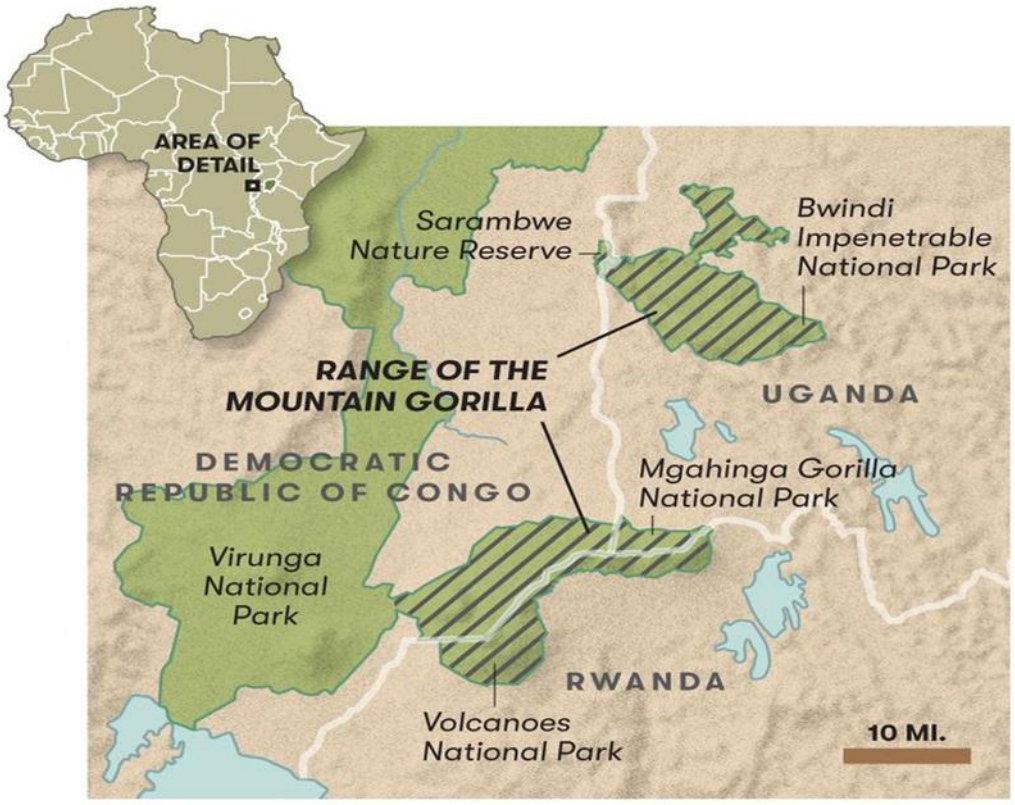

Figure 1: Mountain gorilla distribution. From Gates et al. (2020).

1.2.2. Historical development of mountain gorilla populations

Mountain gorillas were first described as a species in 1903 and classified as endangered by the IUCN red list in 1986 and as critically endangered in 1996 (Hickey et al. 2020). In 1960, the Virunga gorilla population was estimated between 400 and 500 individuals, which 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). The Virunga gorilla population was stable from 1973 to 1978, with an estimated number between 252-285 individuals, and then continuously grew to 380 gorillas in 1989 (Gray et al. 2009). Since then, the population has been increasing with annual growth rates around 3–4% between 2003 and2016 (Granjon et al., 2020; Gray et al., 2013). Research findings showed that populations in the Virunga Massif have grown

from an estimate of 400 in 1980s to 480 in 2010, and 604 individuals in 2016, which together with Bwindi population make the total mountain gorilla number over 1,000 individuals (McNeilage et al. 2006; Robbins et al. 2011; Gray et al. 2013; Hickey et al. 2019).

While population dynamics of the Virunga gorillas have been studied extensively, data from Bwindi are limited. Bwindi experienced lower levels of anthropogenic disturbance than the Virunga population, and Robbins et al. (2009) found out that the Bwindi population has shown little or no growth over the past 20 years. It is assumed that the lower growth rate at Bwindi may arise mainly from lower fertility than the Virunga study groups, rather than higher mortality. They speculated that the more frugivorous Bwindi gorillas have a slower life-history, or that they are closer to the carrying capacity of their habitat. However, results from the last two Bwindi-Sarambwe surveys indicated substantial growth for this population between 2011 and 2018 (Hickey et al. 2019b).

The population increase in Virunga Massif has been almost entirely attributed to the growth rate in habituated groups due to the "extreme conservation" measures these groups benefit from, such as daily group monitoring and protection, veterinary interventions and anti-poaching patrols (Robbins et al. 2011). Also habituated groups at Bwindi had a higher growth rate than the rest of the population, possibly because they receive veterinary care and better protection from poachers (Robbins et al. 2009). In Virunga Massif, the population increase has not been uniform, which may be due to varying ecological conditions that are linked to different habitat types and food availability across the habitat range (Kalpers et al. 2003; Ganas & Robbins 2005; Gray et al. 2013; Hickey et al. 2019b; van del Hoek et al. 2019). Moreover, the potential for spatial expansion is extremely limited due to dense human communities living nearby. Consequently, as gorilla population densities increase, their susceptibility to infectious diseases may also increase (Petrželková et al. 2021). The analyses of mountain gorilla genomes by whole genome sequencing indicated extensive inbreeding, 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).

1.2.3. Current threats to mountain gorilla populations

Due to continuous growth in recent years, IUCN classified mountain gorillas down from a Critically Endangered to Endangered species in 2018, which is a considerable success story owed to the conservation efforts of protecting these animals, which were once expected to be extinct by the end of the twentieth century (Gray et al. 2013). However, there remains an ongoing need for conservation management, with the two remaining populations still facing threats that can drive them to decline or get extinct in their limited habitats.

Mountain gorillas live in the regions with high human population densities with poor livelihoods. Most people depend on agriculture which leads to agricultural land conversion, linked with competitive exploitation for limited natural resources which resulting in deforestation and degradation of mountain gorilla natural habitat (Plumptre et al. 2003; Kanyamibwa 2013). This includes illegal timber extraction, destruction of habitat for charcoal, firewood or farmland, gathering bamboo or small trees for construction materials and human-induced fires to maintain grassland inside parks for illegal grazing (Hickey et al. 2020). In DRC oil exploitation is regarded as one of the powerful resources for economic development, but it can lead to destruction and fragmentation of the endangered mountain gorilla's habitat (Bizawu & Gomes 2016). The government of DRC considered declassifying portions of Virunga National Park for the purpose of oil drilling (Hickey et al. 2020). Thus, mountain gorillas may not be as well protected from exploitation as previously believed.

Although the benefits of regulated tourism and contribution to gorilla conservation have been known (Robbins et al. 2011), the risks are appreciable (Macfie & Williamson 2010). Almost 70% of Virunga gorillas (Hickey et al. 2018) and approximately 40% of Bwindi gorillas (Roy et al. 2014) are habituated for tourism or research. Two hundred sixty four to 880 people come within close proximity of mountain gorillas every day of the year – about 98,360–321,200 people annually. Despite IUCN guidelines recommend to remain >7 m from gorillas (Macfie & Williamson 2010), proximity is usually less (Sandbrook & Semple 2006). The habituation process is stressful to the gorillas (Williamson & Feistner 2011; Shutt et al. 2014) and the risk of pathogen transmission

between humans and gorillas is increased (see below). Habituated gorillas are also more vulnerable to human attacks (Williamson & Fawcett 2008).

The civil wars and political instability in the region have had a direct impact on gorilla populations. Between 1992 and 2000, 4-5 % of the population of mountain gorillas were killed as result of the war around their habitats (Plumptre & Williamson 2001; Kalpers et al. 2003). Moreover, the Mikeno Sector of Virunga National Park has repeatedly experienced problems with rebels and park authorities have had great difficulty monitoring the gorillas here (Sawyer 2018).

Poaching is a serious threat, leading to deaths/injuries of mountain gorillas in the region (Plumptre et al. 2003; UNEP 2003). Poaching of mountain gorillas has been reported since the 1960s, when gorillas were poached primarily for bush meat, as well as for traditional medicine or trade to foreign countries. Illegally exported gorillas can be kept in captivity for human traditional beliefs/medicine or kept as pets and in zoos (Karno 1990; Kalpers et al. 2003). Although there have been no such known cases since 2007 (Williamson & Fawcett 2008), retaliatory killings were documented in addition to gorillas killed during the political instability of the 1990s (Kalpers et al. 2003; Robbins et al. 2011), but in the past decade, only three direct killings were documented (Direkthilfe & Berggorilla 2007). However, mountain gorillas still face poaching threats through falling into snares purposely set for bush meat of antelopes, duikers, bush pigs, and other animals, which leads to injures or death of mountain gorillas (Gray et al. 2009; Kanyamibwa 2013).

The impact of climate change on gorilla habitat was modelled by Thorne et al. (2013) with increased temperatures and modified rainfall patterns predicted, but the variation in modeling outputs needs to be taken in consideration. Those predicted changes may cause changes in food availability and habitat quality for mountain gorillas (Mcgahey et al. 2013), subsequently impacting their ranging patterns and foraging activities (Grueter et al. 2013). On top of that, climate change is likely to have negative effects on the human populations next to gorilla habitats, possibly leading to increased socio-political instability (Plumptre et al. 2017; Shabahat 2018), which can hinder mountain gorilla conservation efforts.

Disease is one of the serious and major threats to non-human primates, including mountain gorillas. Great apes are susceptible to human pathogens due to their genetic relatedness. As most mountain gorillas are habituated to human presence, there is an increased frequency of human contact, which consequently increases the risk of disease transmission (Gilardi et al. 2021). Human pathogens have previously caused disease outbreaks in mountain gorillas, including fatal respiratory virus infections (Palacios et al. 2011) and a measles virus outbreak, prompting vaccination of more than 60 mountain gorillas to reduce morbidity and mortality (Hastings et al. 1991). Respiratory outbreaks in the Virunga gorillas are increasing in frequency, with pathogenic human respiratory viruses repeatedly introduced into mountain gorilla populations from humans, which calls for stringent biosecurity measures to protect gorilla health (Spelman et al. 2013; Mazet et al. 2020) .This need is now increasingly urgent due to the recent COVID-19 pandemics (Gilardi et al. 2021)

Ebola virus disease (EVD) is another disease of particular concern for the long-term survival of mountain gorillas, with infections in humans often fatal and great apes known to be highly. Western lowland gorilla and chimpanzee populations have experienced up to 95% mortality due to the Zaire and Tai Forest strains of EVD (Leendertz et al. 2017). Although mountain gorillas have not experienced any EVD outbreak to date, this virus killed several people in Uganda and an EVD outbreak occurred in North Kivu Province, DRC, in 2018/19 (Towner et al. 2008; Shoemaker et al. 2011; MGVP 2019). Veterinary and government wildlife authorities, together with international partners and frontline healthcare workers, need to closely monitor the situation and develop a plan for protection from and possible mitigation of EVD in mountain gorillas.

The changes in gorilla population dynamics, social structure, and habitat use, caused by growing gorilla populations in a restricted area, may be altering stress levels (Harcourt et al. 2001; Caillaud et al. 2014; Eckardt et al. 2019). This could result in changes of pathogen epidemiology with possible impact on gorilla health. Clinical diseases caused by parasites have been observed in mountain gorillas; post-mortem examinations of 60 gorillas from 1985 to 2007 revealed histopathologic evidence of enteritis in more than 50% of cases, gastritis in more than 35% of cases, and colitis in more than 25% of cases (Muhangi et al. 2021). In the Virunga gorillas severe gastritis caused by strongylid nematodes infections were recorded (Muhangi et al. 2021;

Petrželková et al. 2021), especially in young silverback gorillas ranging between Mounts Karisimbi and Visoke after group densities had significantly increased (Petrželková et al. 2021). Chronic weight loss and declining body condition was observed in adult gorillas across Bwindi NP, which may be attributed, at least in part, to high strongylid infection intensities (Petrželková et al. 2021). Striking geographic differences in both composition and intensities of strongylid infections were revealed across Virunga Massif with higher strongylid egg counts and dominance of pathogenic gastric strongylids mostly in areas with high occurrences of gastrointestinal disease (Petrželková et al. 2021, Petrzelkova et al. unpublished data). More detail study showed that mountain gorillas are at particular risk of high strongylid infection levels in particular areas of the Virunga Massif. These areas are characterized by higher precipitation, lower temperatures and particular vegetation types associated with higher elevations, as well as increased individual densities and consequent reduced home range (Petrželková et al. 2022). In conclusion, new health challenges may be emerging because of the successful conservation of mountain gorillas, as evidenced by the steady increase in gorilla numbers in recent decades but with limited possibility for the gorillas to expand their range.

1.2.4. Parasites of wild gorillas

Wild primate populations, including the African great Apes, host a vast diversity of parasites, with over 400 'parasitic' (*sensu lato*) species previously identified, including viruses, bacteria, helminths and vector-borne diseases (Nunn & Altizer 2005). Within this chapter I will focus only on parasites within a more conventional scope of the parasitology discipline (Schmidt et al. 2009) and restrict to gastrointestinal parasites of both gorilla species. The gastrointestinal parasites of African great apes, including gorillas, can be largely grouped to protists (unicellular eukaryotes) and helminths (multicellular eukaryotes, worm-like parasites). Although traditionally coproscopic examination have been widely used for diagnostic purposes of parasites of non-human primates (Modrý et al. 2018), genotyping and subtyping of particular isolates using various molecular approaches provide the essential information about the diversity and zoonotic potential of gorilla parasites (Sak et al. 2013; Pafčo et al. 2018).

Both species of gorillas are large herbivores and their parasite fauna is similar to other non-ruminant herbivores, dominated mostly by ciliates, strongylid nematodes and

in the case of mountain gorillas also by anoplocephalid tapeworms (Pafčo 2018). Gorillas deposited two- to threefold the amount of feces at nest sites and thus the nest sites may be hotspots for gastrointestinal pathogen transmission through direct contact or when gorillas ingest foods contaminated with infectious larvae during site revisits (Sinayitutse et al. 2021).

Virtually all gorilla individuals harbor entodiniomophid ciliates, which are in fact commensals or even mutualists, actively participating in hindgut fermentation within their hosts (Profousová et al. 2011). In comparison to chimpanzees with only two species of two genera (Pšenková-Profousová 2018), entodiniomorphid communities in gorillas are much more diverse (Costello & Franklin 2009; Pšenková-Profousová 2018; Ito et al. 2020). Recently, an intensive research effort was dedicated to identify the composition of entodiniomophid communities in Virunga mountain gorillas, resulting in finding 13 species of five genera (*Troglocorys*, *Gorilloflasca*, *Prototapirella*, *Troglodytella*, and *Opisthotrichum*) summarized in Ito et al. (2020). Interestingly, wild hindgut fermenting gorillas might have rumen ophryoscolecids in the intestine, sympatric with their native intestinal ciliates (Ito et al. 2020).

Intestinal trichomonads are a neglected part of the gorilla intestinal ecosystem yet may be widely distributed in gorilla hosts. However, with exception of study of (Petrželková et al. 2020), who found host specific genotypes of *Tetratrichomonas* in western lowland gorillas, central chimpanzees and humans co-habiting Dzanga Sangha Protected Areas, information from other gorilla populations is missing.

Intestinal amoebas of the genus *Entamoeba* colonise the digestive system of a wide range of hosts from invertebrates to humans (Hooshyar et al. 2015) including gorillas (Jirků-Pomajbíková & Vlčková 2018). Understanding the complex *Entamoeba* communities in the gorilla host was significantly advanced by application of high-throughput sequencing on samples of sympatric western lowland gorilla, central chimpanzee and humans in Dja Faunal Reserve, Cameroon (Vlčková et al. 2018). Most of the haplotypes detected in Vlčková et al. (2018) belonged to commensal *Entamoeba* species; however, the pathogenic species (*Entamoeba histolytica* and *Entamoeba nuttalli*) were also detected. Other amoebas identified to date includes *Endolimax nana* and *Iodamoeba buetschlii* (Rothman & Bowman, 2003).

Several studies showed high prevalence of asymptomatic microsporidial infection in gorillas (Graczyk et al. 2002b; Sak et al. 2013, 2014), while *Cryptosporidium* and *Giardia* spp. seems to be a less frequent pathogen (Nizeyi et al. 1999; Graczyk et al. 2002b; Gillespie et al. 2009). It was assumed that occurrence or increased prevalence of these pathogens could be a result of frequent presence of humans and livestock in gorilla habitat (Nizeyi et al. 2002a; Salyer et al. 2012), rather than resulting from close contact with humans (Sak et al. 2013). From other protists, *Blastocystis* spp. and *Chilomastix mesnili* were identified in gorillas (Rothman & Bowman 2003) , while *Balantioides coli* is rarely found in wild gorillas, while very common in captive ones, although the source of infection has not been clarified (Pomajbíková et al. 2010).

Strongylid nematodes are likely the most prevalent helminths in gorillas with infections documented across multiple localities (Rothman & Bowman 2003; Kalema-Zikusoka et al. 2005; Makouloutou et al. 2014; Pafčo et al. 2018; Mason et al. 2022). The spectrum of strongylid nematodes infecting gorillas is wide, with nematodes from various strongylid genera reported: *Oesophagostomum*, *Necator*, *Hyostrongylus*, *Paralibyostrongylus*, *Murshidia*, *Impalaia*, *Trichostrongylus* and *Mammomonogamus* (Mason et al. 2022). Although gastrointestinal helminthiases are typically asymptomatic in wild non-human primates, host factors like immune status, or extrinsic factors, especially those caused by human activities like e.g. habitat loss, fragmentation and other anthropogenic pressures can alter the transmission dynamics of helminths. Changing pathogen transmission may increase host susceptibility and consequently exacerbate negative effects of infections on health, as observed in the case of strongylid nematodes in mountain gorillas (see chapter 1.2.3), while no symptoms have been observed in wild western lowland gorillas (Mason et al. 2022).

Several other nematodes also infect African great apes, such as *Strongyloides* spp., with the human nematodes *S. fuelleborni* and *S. stercoralis* identified (Bradbury et al. 2021), though human-ape transmission lacks strong molecular evidence (Hasegawa et al. 2016). In wild gorillas, nematode from genus *Ascaris* and *Trichuris* are reported infrequently in comparison to captive gorillas, which probably get infected from humans or other non-human primates sharing the captive facilities (Pafčo 2018). *Capillaria*-like nematodes have been described in mountain gorillas (Graczyk et al. 1999).

Various species of *Probstmayria* have also been described in African great apes and while probably common in wild populations, current knowledge is scarce due to improper diagnostic methods (Freeman et al. 2004; Rothman et al. 2008). While gastrointestinal spirurid nematodes are reported in great apes, they are commonly documented as "spirurid eggs" due to differentiation of the described species, and even genera, difficult through light microscopy (Pafčo et al. 2018). Though, two species are known to infect gorillas, namely *Chitwoodspirura wehri* and *Protospirura muricola* (Hasegawa 2018).

Gorillas also host two genera of anoplocephalid tapeworms, namely *Anoplocephala* and *Bertiella* (Doležalová 2018). Our understanding of their taxonomy is limited, due to infections being diagnosed through presence of eggs and proglottids in fecal samples, with taxonomically important structures, such as scolexes, often unavailable. *Anoplocephala gorillae* is commonly identified in mountain gorillas, with infection linked to ingestion of mites, the suspected intermediate host (Proudman et al. 1997). High number of adult *Anoplocephala gorillae* have been observed during necropsies of Virunga mountain gorilla (Petrželková et al. 2021), but with probably no clinical impact (Muhangi et al. 2021). Whereas tapeworm infection of lowland gorillas is infrequent and documented as *Bertiella* sp. (Pafčo et al. 2018).

Our knowledge of trematode infections in gorillas is extremely limited, caused by indistinguishability of the eggs used for identification of infections when non-invasively sampling primates. Egg detection combined with molecular work confirmed *Schistosoma mansoni* infection in lowland gorillas and chimpanzees, a causative agent of schistosomiasis in humans (Červená et al. 2016).

1.2.5. Microsporidia

Classification: Microsporidia are known as a spore-forming unicellular parasites causing microspordiosis disease. Previously ranked as protozoa or protists, they have been reclassified and now are known as a part of fungi (Hibbett et al. 2007) with up to 1500 identified microsporidium species divided to 200 genera (Winters & Faisal 2014; Bojko et al. 2022). Microsporidia infect both a large variety of invertebrates and vertebrates including domestics, livestock animal and non-human-primates and human

(Graczyk et al. 2002a; Sak et al. 2014). Based on their structural characteristics, microsporidia categorized into three primary types. The first group known as the primitive group, deprived with a polaroplast and has an underdeveloped polar filament, the second group is the intermediate class that include Chytridopsidae, Hesseidsae and Burkeidae which characterized by the short polar filament, a developing polaroplast and endospore (Han & Weiss 2017). The third group is known as the higher microsporidia is characterized be a fully developed polar filament, polaroplast and posterior vacuole. The classification of microsporidia is mainly based on spore morphology, namely size of the spore, number of polar coils with the spore, configuration of the nuclei in the spore, supplemented with their rRNA genetic analysis or gene sequencing. Microsporidia are characterized by a fully developed polar filament, polaroplast and posterior vacuole (Cali et al. 2017; Bojko et al. 2022)

Description: They are obligate unicellular parasites replicating solely in host cells, forming egg-shaped spores (Figure 2), measuring 1-40 µm depending on the species that are environmentally resistant infective stage. Under the spore wall, the plasmatic membrane encloses organelles situated inside the spore, such as sporoplasm, nucleus, endoplasmic reticulum, posterior vacuole, and ribosomes. The characteristic set of organelles in microsporidia spores is so-called extrusion apparatus consisting of coiled polar tube, membranes of polaroplast and anchoring disc serving for the inoculation of infectious sporoplasm into new host cell. The number and arrangement of polar tube coils is species specific (Winters & Faisal 2014). The transmission is mostly fecal-oral, but transplacental, inhalation or ocular infections were described as well. Thus, ingestion spores in contaminated food and water, contact with infected animals or persons and their feces or urine, inhalation of contaminated aerosols belong to major routes of infection.

***Diagnostics*:** Spore of microsporidia can be detected in fecal, biopsy and necropsy specimens, urine sediment and other body fluids (Figure 2). The most common detection method is microscopy observation of fecal smears following by staining with chromotope 2R-Based stains, chemifluorescent optical brightening agents, or Uvitex 2B. Fluorescein-tagged antibodies can be used to detect spores in smears. The microsporidia can also be detected by electron microscopy or histology examination of fixed tissue, which requires biopsy or necropsy material. Tissue for histology can be stained with modified chromotrope 2R, Tissue Gram stain, or Luna stain. Detection of specific DNA of

microsporidia by PCR/RT-PCR is rapid and affordable. Electron microscopy, advanced immunoassay, or molecular tools are necessary for identification of species. It should be noted that micropsordia spores are excreted intermittently (2-14 days) in feces and urine (Han et al. 2021).

***Life cycle*:** The spores are the infective form of the microsporidia (Figure 2). Once ingested or inhaled, the spores germinate in appropriate conditions of host bodies and activation process leads to extrusion of polar tube and injection of sporoplasm through its lumen into a cytoplasm of a new host cell. Inside the cell, the sporoplasm grows into a meront, which undergoes extensive multiplication, transform to sporonts which mature into spores (Lallo & Paulista 2014). Spores are shed into the environment mainly in the feces and urine (Hibbett et al. 2007; Han & Weiss 2017). At the infection stage, various microsporidia species invade all organs and tissues, and some of them are lethal (Hibbett et al. 2007; Winters & Faisal 2014).

Pathogenicity and clinical importance: Microsporidia are obligate intracellular parasites causing severe infections with lethal outcome in immunocompromised hosts. They are considered opportunistic pathogens, because they primarily cause overt disease when the host's immunity is reduced and so the parasite can easily overspread. Microsporidia destruct the host cell completely and abuse its metabolism for own reproduction. The replication of microsporidia takes place within the host's cells and infect by means of unicellular spores through ingestion spore in food and water, fecal-oral, inhalation, direct contact with the conjunctiva, animal contact and from one person to another (Winters & Faisal 2014). The alimentary tract is a common target tissue for the production of infective spores, and infection can be restricted to one portion of the gut or include several tissue types. Systemic microsporidian infections acquired by ingesting spores are also initiated in the gut epithelial cells followed by the invasion of other tissues.

Fifteen species are current identified to be pathogenic to human including *Enterocytozoon bieneusi*, *Encephalitozoon* species (*E. cuniculi, E. hellem, E. intestinalis)*, *Anncaliia* (*A. algerae*, *A. connori*, *A. vesicularum*), *Microsporidium* (*M. ceylonensis*, *M. africanum*), *Trachipleistophora* (*T. hominis, T. anthropophthera*), *Nosema ocularum***,** *Pleistophora ronneafiei*, *Vittaforma corneae*, *Tubulinosema acridophagus* (Winters & Faisal 2014). *E. bieneusi* and *Encephalitozoon*

spp. infect humans including children, diabetes patients and patients with HIV infection, they occur in severely immunodeficient humans and result in the symptoms like diarrhea, anorexia, weight loss and fever (Němejc et al. 2014; Sak et al. 2014). Microsporidia infections in human are assumed to be zoonotic, mostly transmitted indirectly via of contaminated food or drink water and directly through close contact of infected humans and animal, with domestic and farm animals serving as reservoirs (Didier et al. 2004; Mathis et al. 2005; Santín & Fayer 2011). It was argued that some species of microsporidia usually infect insects, which are intermediate hosts for the transmission of different diseases to crustaceans and fish, and up to 10% of the species were found to be parasites of vertebrates including humans (Sak et al. 2008) and birds with the infections being asymptomatic (Saková et al. 2006).

The molecular studies detected the presence of *E. bieneusi* and *Encephalitozoon* spp. in free ranging and captive populations of apes (Sak et al. 2013, 2014). The prevalence of microsporidia is highest in apes kept in zoos in comparison to wild (Sak et al. 2011c, 2013, 2014). Clinical disease or pathological findings have not been reported in apes. The clinical impact is unknown, but it is suggested that the course of infection is similar to human. *E. hellem*, *E. cuniculi*, *E. intestinalis* and *E. bieneusi* were reported to infect domestic animal (cats and dogs), free ranging animals and livestock (Sak & Kváč 2018). These four microsporidia species have been identified as species mostly infecting both humans and apes, and are commonly found in domestic animals ranging near apes' habitats or having contact with captive and habituated great apes (Sak et al. 2013; Mynářová et al. 2016).

Occurrence in the great apes: Approximately 1500 microspordia species and 200 genera of microspordia, 15 are infectious for humans. Out of these, the following species have been found in non-human primates (Sak & Kváč 2018):

- *E. cuniculi* in chimpanzee (*Pan troglodytes*), bonobo (*P. paniscus*), mountain gorilla (*G. b. beringei*), western lowland gorilla (*G. g. gorilla*), Bornean orangutan (*Pongo pygmaeus*), Sumatran orangutan (*P. abelii*)
- *E. intestinalis* in western lowland (*G. g. gorilla*), mountain gorilla (*G. b. beringei*), Bornean orangutan (*Pongo pygmaeus*), Sumatran orangutan (*P. abelii*)
- *E. hellem* in chimpanzee (*P. troglodytes*)

- *E. bieneusi* in mountain gorilla (*G. b. beringei*), western lowland gorilla (*G.g. gorilla*), chimpanzee (*P. troglodytes*), bonobo (*P. paniscus*), Bornean orangutan (*P. pygmaeus*), Sumatran orangutan (*P. abelii*), northern white-cheeked gibbon (*Nomascus leucogenys*)

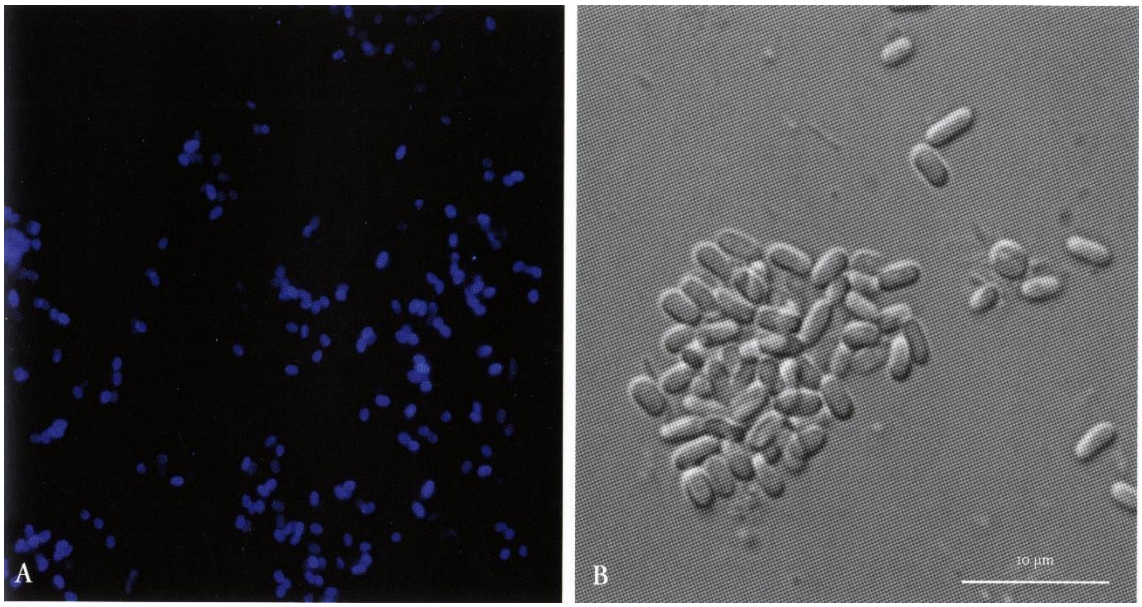

Figure 2: **Spores of *Encephalitozoon cuniculi*. Spore of *E***. *cuniculi* from tissue culture in smear stained optical calcofluor visualized using UV light microscopy. B) Spores of the same species obtained from tissue culture under differential interference contrast (DIC). From Sak & Kváč (2018).

1.2.6. *Cryptosporidium* spp.

Classification: The *Cryptosporidium* genus belongs to the phylum Apicomplexa. Cryptosporidia were previously misclassified as coccidia based on oocyst morphology and host development (Fayer et al. 1997), but detailed studies based on developmental stage morphology and molecular analyses (Carreno et al. 1999; Valigurová et al. 2007) have shown that cryptosporidia are distinct from Coccidia and have been reclassified to the subclass Cryptogregarina within the class Gregarinomorphea (Cavalier-Smith 2014). To date, 49 valid species as well as several dozen genotypes of *Cryptosporidium* have been described, distinguished based on morphological differences, host specificity or

developmental cycle localization, and molecular differences (Fayer 2007; Kváč et al. 2013a, 2013b, Kváč et al. 2014b).

Host specificity: Host specificity encompasses the range and diversity of host species that a parasite is capable of infecting and is considered a crucial measure of a parasite's potential to shift hosts and trigger disease emergence (Wells & Clark 2019). *Cryptosporidium* is known as protozoan parasite infecting a gastrointestinal epithelial cell of many classes of vertebrates including amphibians, reptiles, fish, bird and mammals (Appelbee et al. 2005; Salyer et al. 2012; Parsons et al. 2015). According to host specificity, *Cryptosporidium* spp. are divided into species and genotypes with high host specificity (oioxenous), with the ability to parasitize several phylogenetically related hosts (stenoxenous), and with the ability to parasitize multiple unrelated host species (euryxenous). The group of oioxenous cryptosporidia is small and includes, for example, *C. wrairi*, which parasitizes guinea pigs, or *C. parvum* subtype IIc, which exclusively infects humans (Vetterling et al. 1971; Kváč et al. 2014b). Most known cryptosporidia are stenoxenous. The group of euryxenous cryptosporidia, representatives with very low host specificity, includes the species *C. parvum*, *C. baileyi* and *C. ubiquitum* (Kváč & McEvoy 2018a).

Life cycle: *Cryptosporidium* spp. infect the host through oocysts (Figure 3). Oocysts are endogenous resistant forms of the parasite that can withstand harsh environments until they are ingested by another host (Leav et al. 2003; Ghazy et al. 2015). *Cryptosporidium* oocysts pass in the feces of an infected person or animal and can be easily transmitted in many ways by the contact with animals, especially calves and goats, and their environment, having contact with infected people, and indirectly by drinking water and eating foods contaminated with *Cryptosporidium* spp. Oocysts (Ryan et al. 2014; Ghazy et al. 2015; Parsons et al. 2015). *Cryptosporidium* spp. have monoxenous life cycle which involves only one host and they are able to undergo both asexual and sexual reproduction, which may suggest an easy transmission from one individual to another. The oocyst excysts to release four sporozoites, which invade epithelial cells of the gastrointestinal tract, develop into a trophozoite, and undergo asexual replication (merogony) to produce eight merozoites (type I meronts). Merozoites invade new cells to form type I meronts, repeating the asexual cycle, or type II meronts, which release four merozoites that enter the sexual cycle (gametogony) by producing either a microgamont

(male stage) or macrogamont (female stage). The microgamont releases many microgametes, which and fuse with uninucleate macrogametes to form a zygote. The zygote undergoes sporogony leading to production of four sporozoites. Oocysts sporulate *in situ* and are infectious immediately after defecation (Kváč & McEvoy 2018a).

Description: Sporulated oocysts generally measure 4.5 × 5.5 μm in species inhabiting the intestine and 6.0 × 8.0 μm in species inhabiting the stomach (Figure 3). Although there is a variability in oocyst size among the species, differences are generally small, and oocyst size is not of practical significance for identification. During the microscopy following flotation technique, the oocysts can be seen only in case of severe infection with high oocyst output (> 2000 oocyst per gram of feces).

Diagnostics: *Cryptosporidium* spp. infections are diagnoses by examination of stool samples, microscopy can be used to detect stained or immunolabeled oocysts in fecal smears by using different techniques (e.g., acid-fast staining, cold Kinyoun, aniline-carbol-methyl violet, Ziehl–Neelsen, fluorescent staining with auramine, or direct fluorescent antibody). The detection threshold is 2000 and 1000 oocysts per gram (OPG) for staining and immunolabeling. Flotation method (e.g. Sheather's sugar flotation and zinc sulfate flotation) is useful to concentrate oocysts but a sensitivity varies among different flotation methods. An enzyme immunoassays can detect up to 500 OPG, although not all species can be detected. The PCR approach can detect 20 OPG and is the only approach that can differentiate among species and genotypes.

Pathogenicity and clinical importance: The immune system of the potential *Cryptotosporidium* host plays an important role in the development and progression of the infection. The main symptoms of cryptosporidiosis include watery diarrhea, often with mucus; blood in the stool is rare. Other clinical signs include nausea, cramps in the epi- and mesogastrium, and fever (Jokipii & Jokipii 1986). The people, who are extremely undernourished, young children and those with concurrent health problems, such HIV/AIDS patients, are at a higher risk of contracting cryptosporidiosis (Salyer et al. 2012; Sak et al. 2014). Very little is known about cryptosporidiosis in wildlife (Appelbee et al. 2005). Most of the work published to date, based on field observations, does not address the clinical manifestations of infection in wildlife. Knowledge of *Cryptosporidium* infection and cryptosporidiosis in great apes remains relatively poor

(Sak et al. 2013, 2014). *Cryptosporidium* infections may affect the apes similarly to humans. *Cryptosporidium* infection could be highly associated with diarrhea or gastrointestinal dysfunction. However, no clinical signs of cryptosporidiosis were also observed in *Cryptosporidium* positive apes.

Occurrence in the great apes: Out of the 49 species and more than 100 genotypes of *Cryptosporidium*, 22 are infectious for humans. Out of these, the following *Cryptosporidium* species has been found in apes (Kváč & McEvoy 2018a):

- *C. parvum* in mountain gorilla (*G. b. beringei*), Bornean orangutan (*P. pygmaeus*)
- *C. bovis* in western lowland gorilla (*G. g. gorilla*)
- *C. muris* in western lowland gorilla (*G. g. gorilla*), Bornean orangutan (*P. pygmaeus*), Sumatran orangutan (*P. abelii*)
- *C. meleagridis* in western lowland gorilla (*G. g. gorilla*)
- *C. suis* in eastern chimpanzee (*P. t. schweinfurthii*)
- *C. hominis* in eastern chimpanzee (*P. t. schweinfurthii*)

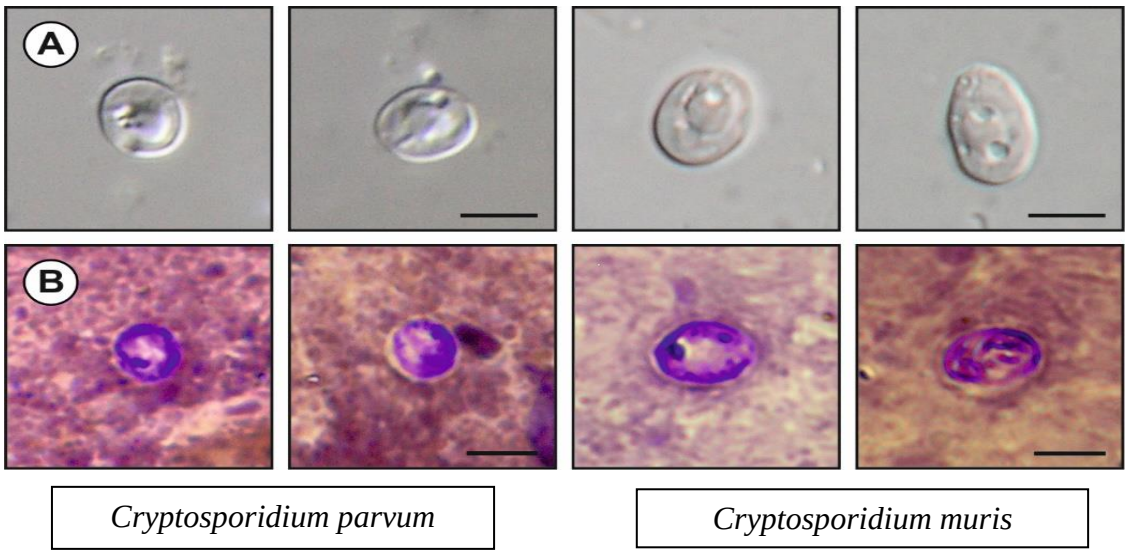

Figure 3: Morphology of the *Cryptosporidium* oocyst. A) *Cryptosporidium* unstained oocysts photographed with differential interference contrast (DIC microscopy). B) *Cryptosporidium* oocysts stained by aniline-carbol-methyl violet. From (Kváč & McEvoy 2018a).

1.2.7. *Giardia intestinalis*

Classification and distribution: *G. intestinalis* (also known *G. lamblia* or *G. duodenalis*) is a flagellated protozoan that causes giardiasis. It is classified in the phylum of Metamonada, order of Diplomonadida in the family of Hexamitidae. *G. intestinalis* is categorized into eight genetic groups known as assemblages (A to H) based on the host species (Appelbee et al. 2005;; Ahmed et al. 2019). The assemblages A and B are considered as zoonotic and they are known to infect humans and non-human primates and have been identified in gorillas (Nizeyi et al. 2002b; Sak et al. 2013). Previous research showed that assemblages A and B can be found in some reservoir hosts like dogs, cats, wildlife and livestock (Feng et al. 2011). Other assemblages infect other domestic and wild animals (Thompson et al. 2008). The assemblages C and D are identified in domestic and wild canines, the assemblage E is reported frequently in ruminants and pigs, but not widespread in humans, F in cat, G in rodents and H is frequently reported in seals and gulls. However, assemblage C, D, E and F can be also found in human (Appelbee et al. 2005; Xiao & Feng 2017; Dixon 2021).

Description: Cysts are oval, measuring 8-12 × 7-10 μm, with thin refractile wall (Figure 4). The mature cysts contain two trophozoites with two nuclei each. Trophozoites are bilaterally symmetrical (9-21 × 5-16) have four pairs of flagella and an adhesive disc. Both cysts and trophozoites are diagnostic stages.

Diagnostics: Giardiasis is diagnosed through the microscopical examination of wet mounts or concentrations samples to look for cysts (Figure 4) or trophozoites in stool specimens. Trophozoites are found primary in diarrheal feces and can be observed easily in stained smear (trichrome staining) or wet mount. A wet mount allows trophozoites motility to be observed. Trophozoites are not generally present in formed feces(Dixon 2021). Cysts are excreted intermittently, so it is necessary to examine several fecal samples over multiple days (three samples collected every second day). Cysts are identified by microscopy in any type of the feces. Cysts may be concentrated by various flotation techniques but tend to shrink and become distorted beyond recognition observed under microscopy. The phase-contrast microscopy is helpful for identification. If only bright-field microscopy is available, a drop of Lugol's solution can be added to the sample slide to enhance to contrast of nuclear staining in cysts and trophozoites. Other tools

include immunofluorescence staining, fecal immunoassays and PCR which is based primary on the amplification of ß-gardin, GDH and/or TPI gene.

Life cycle*:* Life cycle of *G. intestinalis* is direct, the infective cysts are shed in feces and transmitted via fecal-oral route in contaminated food or water. Excystation take place in duodenum, releasing two trophozoites from each cyst. Trophozoites reproduce asexually by longitudinal binary fission and later encyst in the small intestine. Cysts shed in feces are immediately infectious. The asexual reproduction is the most dominant pathway of the spread amongst populations with a huge impact in the young/children and domestic animals (FAO 2014). Thus, easy transmission of giardiasis is facilitated by their simple and direct life cycle (Thompson et al. 2008; Schnell et al. 2016; Thompson & Ash 2016).

Pathogenicity and clinical importance: *G. intestinalis* has a diversified range of hosts; with vertebrates being the most vulnerable. They are easily transmitted from one individual to another through ingestion of cysts in contaminated food by their environmentally resistant infective stages, by contact with the contaminated materials, or contact with animal or human suffering from giardiasis (Choy et al. 2014). Trophozoites do not survive in the environment after their excretion in the feces. Limited studies suggest that captive animals and wild animals under human pressure are more frequently infected than wild animals without human contact (Nizeyi et al. 1999; Sleeman et al. 2000; Gillespie et al. 2009; Berrilli et al. 2011; Kváč et al. 2014a). Limited space in zoos and sanctuaries and the ability of cysts to persist in the environment for long periods could contribute to higher prevalence of infection in captive animals.

G. intestinalis is globally distributed in humans with billions of annually records, and known to be the intestinal parasite in some regions such as in the United States (Schnell et al. 2016). Giardiasis have been reported as zoonotic disease. The infection may be asymptomatic, depending on the infection period in human and domestic animals, and the symptoms at the later infection period include abdominal pain, nausea, and vomiting, diarrhea, and dehydration (FAO 2014, Thompson et al. 2008).

Occurrence in the great apes: following assemblages of *G. intestinalis* has been found in apes (Kváč & Mc Evoy 2018b).

- assemblage A in western lowland gorilla (*G. g. gorilla*)

- assemblage B in mountain gorilla (*G. b. beringei*), chimpanzee (*P. troglodytes*), northern white-cheeked gibbon (*N. leucogenys*) and white handed gibbon (*H. lar*)

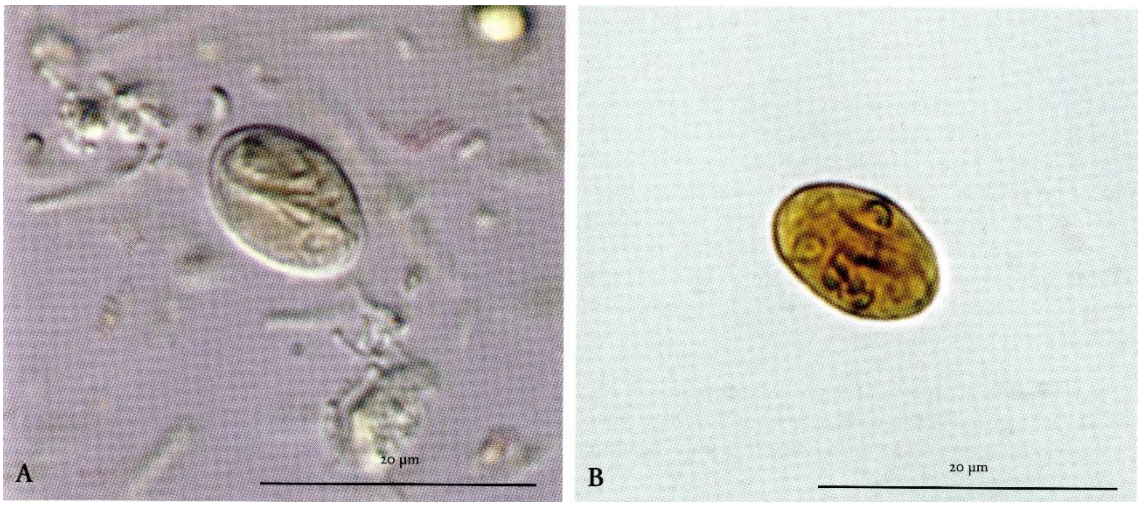

Figure 4: Cysts of *Giardia intestinalis* in the chimpanzee fecal sample. A) Merthiolate-Iodine Formaldehyde (MIF) with NIC microscopy. B) MIF stained with Lugol' solution. From (Kváč & Mc Evoy 2018b).

2. Aims of the thesis

The main goal of this study is to assess patterns of *Cryptosporidium*, *Giardia* and microsporidia infections in endangered mountain gorilla across the entire Virunga Massif. Specific aims have been determined as follows:

- Determine the molecular diversity of *Cryptosporidium*, *Giardia* and microsporidia infections in Virunga gorillas,
- Compare the results with the previous study (Sak et al., 2014) to assess possible temporal changes in these protist infections in last ~ 10 years
- Assess geographical differences in *Cryptosporidium*, *Giardia* and microsporidia infections in gorillas within the Virunga Massif
- Evaluate the impact of sex and age on these protist infections

3. Methods

3.1. Study area description

The study was conducted across the entire range of the mountain gorilla population inhabiting the Virunga Massif, which spans three national parks, namely Volcanoes National Park (NP) in north-western Rwanda, the Mikeno sector of the Virunga NP in the North Kivu Province, eastern in DRC and Mgahinga Gorilla NP in south-western Uganda (Figure 5). The Virunga Massif ranges 451 km^2 in area and is characterized by altitude from 2000 to 4500 meters above sea level (MASL) (Mcneilage 2001). This area has different vegetation types depending on elevation, including the alpine zone (above 3,600 MASL), sub-alpine zone (3,200–3,600 MASL), *Hagenia – Hypericum* zone (2800–3200 MASL), lower altitude forest (mixed forest) (1,600–2,500 MASL), bamboo zone (2500–2800 MASL), disturbed woodland (2300–2800 MASL), open grassland and swamp (Watts 1998; Owiunji et al. 2005). There are two rainy seasons (March–May and September–November) and two dry seasons (December–February and June–August). The most common vegetation type in Virunga NP is the mixed forest, while in Volcano NP it is bamboo, followed by *Hagenia—Hypericum* woodland (Owiunji et al. 2005, Figure 5). A single small section of lower altitude forest remains between Mt. Gahinga and Sabyinyo. MGNP is dominated by mixed forest and bamboo (Figure 5). In the 1950s and 1960s most of the mixed forest in Volcanos NP was destroyed by humans, which pushed the gorilla population to higher elevations, where temperatures can drop to 0°C.

For the purpose of this study, the Virunga Massif was divided into four areas with respect to the volcano positions according to Petrželková et al. (2021). This geographic division was use as a proxy, which reflected observed variation in the occurrence of gastrointestinal diseases, as well as differences in vegetation types, and in historical subpopulation growth and associated current social structure across the Virunga gorilla distribution range (Petrželková et al. 2021). The areas were namely: Karisimbi-Visoke (K_V): Volcanoes National Park, Visoke-Sabyinyo (V_S): Volcanoes National Park,

Sabyinyo-Muhabura (S_M): Volcanoes National Park and Mgahinga Gorilla National Park, Mikeno (M): Virunga National Park (Figure 5).

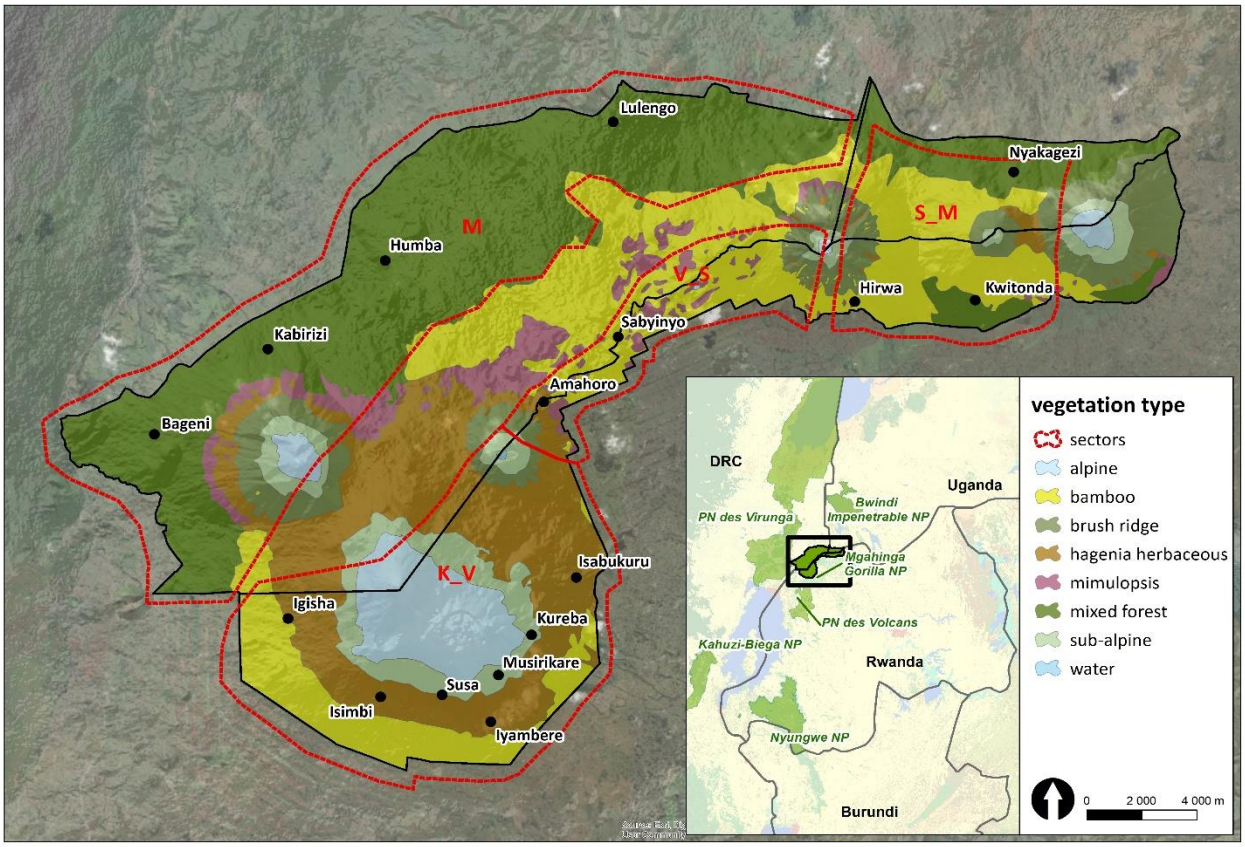

Figure 5: Division of the Virunga Massif into four areas according to volcano positions with location of studied groups. Mikeno; K_V: Karisimbi–Visoke; V_S: Visoke–Sabyinyo; S_M: Sabyinyo–Muhabura). The map is based on Figure 1 from Petrželková et al. (2021) and was created by using ArcGIS Desktop 10.6 (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), boundaries of protected areas were derived from ProtectedPlanet.net database.

3.2. Sample collection

One hundred fifty two fecal samples from 16 monitored gorilla groups were selected from the set of the nest samples collected in 2018 by DFGF, MGVP, RDB, Virunga NP and Uganda Wildlife Authority staff. Only samples from the September/October 2018 were included in (Figure 5; Petrželková et al. (2021). The samples were collected in the morning from gorilla nests of the previous night, put into plastic bags, labeled with group name, collection day and age/sex class based on the estimation of the size of fecal lobes (silverback > 12 years, adult > 8 years, subadult/juvenile > 3.5–8 years, infant 0–3.5 years (Petrželková et al. 2021; Sinayitutse et al. 2021). The groups were assigned to particular areas in Virunga (see above for division) based on 2018 GPS points recorded daily by staff of MGVP, RDB and DFGFI in Rwanda and by MGVP in DRC. There was only one group ranging in Uganda. The collection of fecal samples was non-invasive and did not cause any harm or distress to the animals. The collected samples were transported to the MGVP laboratories in Musanze (Rwanda), Kisoro (Uganda) and Goma (DRC), stored in a refrigerator and within a maximum of 48 h from the estimated defecation preserved in 96% ethanol and stored at -20°C. The samples were later transported to the Czech Republic for molecular analyses of parasites, where they were stored at -20°C.

3.3. DNA isolation

The fecal samples were placed in the thermoblock overnight at 56°C to evaporate the ethanol. DNA was extracted from 200 mg feces by bead disruption for 60 s at 5.5 m/sec. using 0.5 mm glass beads in FastPrep®24 instrument (MP Biomedicals, CA, USA) followed by DNA isolation/purification using a commercially available kit in accordance with the manufacturer's instructions (ExgeneTM stool DNA mini, GeneAll Biotechnology Co. Ltd, Seoul, Korea) by following the steps according to the protocol.

Procedure description:

1. By using pipette discard ethanol and take 200 mg of stool sample to 2 mL microcentrifuge tube (Labelled).

2. Dry open microcentrifuge tubes overnight at 56°C in a termoblock (to evaporate all ethanol from the sample).
3. Rehydratation of the sample: add 1.3 mL of Buffer FL and resuspend the pellet by pipetting up and down. Add the 0.5 mm glass beads and 1 mm zirconia beads to each sample. Rehydrate the sample for several days at 4°C in a refrigerator.
4. Use the Fast-Prep Instrument to bead-beat the samples for 1 min at a speed of 5.5 m/sec.
5. Stand the tube at room temperature for 5 min and put in miniSpin centrifuge for 5 min at a speed of 16,000 *g*.
6. By using the 1,000 μL micro-pipettor, transfer maximum volume of supernatant to a EzPass™ (white). Maximum is 700 μL, minimum 500 μL.
7. Centrifuge at 16,000 *g* for 1 min at the room temperature (miniSpin centrifuge).
8. Discard the supernant solution, which was in collection tube and then reinsert the white mini column back into the same tube.
9. By using 200 μL micro-pipettor, add 100 μL of Buffer EB (Elution buffer) to EzPass™ filter.
10. Incubate for 1 min at room temperature.
11. Centrifuge at 12,000 *g* for 1 min at room temperature (in miniSpin centrifuge)
12. After removing from miniSpin centrifuge reject the white column and remain down tube with EB solution.
13. By using 1,000 μL micro-pipettor, add 500 μL of PB to remain down tube with EB solution then mix well by using pipette up and down and remember to change pipette to every sample.
14. Transfer the mixture to a column Type G (green).
15. Centrifuge at 12,000 *g* for 1 min at room temperature (in miniSpin centrifuge). Discard the pass-through and reinsert the green mini spin back into the same tube.
16. By using 1,000 μL micro-pipettor, add 500 μL of NW to mini column.
17. Centrifuge at 12,000 *g* for 1 min at room temperature (in miniSpin centrifuge). Discard the pass-through and reinsert the mini column back into the same tube.
18. Centrifuge again at maximum speed for 1 min at room temperature (in miniSpin centrifuge) to remove residual wash buffer (drying) then insert the mini column into new 1.5 mL microcentrifuge tube.

19. Add 200 μL of Buffer EB to the center of membrane in the mini column to remove DNA from the column. Centrifuge at 12,000 *g* for 1 min at room temperature.
20. Reject the green minispin column and close the labelled 1.5 mL microcentrifuge tube with isolated DNA.
21. Purified DNA can be either put into the fridge to be used in PCR soon or long-term stored in the freezer at -20°C.

3.4. Nested PCR amplification

All fecal samples were examined for presence of specific DNA of *Encephalitozoon* spp., *Enterocytoozon bieneusi*, *Giardia intestinalis*, and *Cryptosporidium* spp., at the 16S rRNA (small subunit of rRNA), ITS (Internal Transcribed spacer), TPI (triosephosphate isomerase) and, 18S rRNA (small subunit of rRNA) gene loci, respectively.

Procedure description:

1. Defrost PCR reagents at the room temperature.
2. Prepare the PCR master mixture solution by adding all the PCR reagents in a 1.5 mL micro centrifuge tube as suggested in appropriate PCR target reaction (see below).
3. Mix well by vortexing briefly and centrifuge the tube for 5 s to remove any solution trapped in the tube cap.
4. Add 28 μL of the PCR master mixture prepared above to each 0.2 mL PCR tube.
5. Add 2 μL UV-treated distilled water to the tube designated as the blank negative control or add 2 μL PCR product of primary PCR negative control to the secondary PCR tube designated as secondary PCR negative control.
6. Add 2 μL of the unknown/tested DNA samples to the respective tubes with master mixture for primary PCR or add 2 μL of the primary PCR products to the respective tubes with master mixture for secondary PCR.
7. Add 2 μL positive control DNA to the tube designated as primary PCR positive control or add 2 μL PCR product of primary PCR positive control to the secondary PCR tube designated as secondary PCR positive control. The positive controls should not be handled until this step to avoid cross-contamination.
8. Mix the sample tubes by centrifuging briefly.

9. Select the cycling program for the appropriate PCR reaction
10. Start the thermal cycler.

3.4.1. *Enterocytozoon bieneusi*.

The nested PCR protocols described by Buckholt et al. (2002) were used for amplification of sequence of ITS [the primary primers EBITS3 (5′ - GGT CAT AGG GAT GAA GAG - 3′), EBITS4 (5′ - TTC GAG TTC TTT CGC GCT C - 3′); the secondary primers EBITS1 (5′ - GCT CTG AAT ATC TAT GGC T - 3′), EBITS2.4 (5′ - ATC GCC GAC GGA TCC AAG TG - 3′)]. The PCR mixtures contained 2 µL of template DNA, 2.5 U of Taq DNA Polymerase, 1×PCR buffer, 3 mM $MgCl_2$, 200 µM each deoxynucleoside triphosphate, 100 mM each primer and 2 µL BSA in 30 µL reaction volume. DNA of *E. bieneusi* genotype PtEb IX and molecular grade water were used as positive and negative controls, respectively. For both PCR steps, there were total number of 35 cycles, each consisting of 94°C for 45 s, 57°C (for primary PCR) / 55°C (for secondary PCR) for 45 s, and 72°C for 60 s. Initial incubation was at 94°C for 3 min, final extension was at 72°C for 7 min and final soak at 4°C..

3.4.2. *Encephalitozoon* spp.

The nested PCR protocols described by Katzwinkel-Wladarsch et al. (1996) and De Bosschere et al. (2007) were used for amplification of partial sequence of 16S rRNA using *Encephalitozoo*n spp.-specific primers [the primary primers M2F (5′ - CGG AGA GGA AGC CTT AGA GA- 3′) and MFNest (5′ - GAG AGA TGG CTA CTA CGT CCA AGG- 3′); the secondary primers M2R (5′ - ATA GTG ACG GGC GGT GTG T- 3′) and MSP1R (5′ - ACA GGG ACM CAT TCA- 3′)]. The PCR mixtures contained 2 µL of template DNA, 2.5 U of Taq DNA Polymerase (Dream Taq Green DNA Polymerase, Thermofisher Scientific, Waltham, MA, USA), 1× PCR buffer, 3 mM $MgCl_2$, 200 µM each deoxynucleoside triphosphate, 100 mM each primer and 2 µL non-acetylated bovine serum albumin (BSA; 10 $mg.ml^{-1}$; New England Biolabs, Beverly, MA, USA) in 30 µL reaction volume. DNA obtained from spores of *E. intestinalis* grown in vitro in VERO E6 was used as a positive control, and ultrapure water used as a negative control. For both PCR steps a total number of 35 cycles, each consisting of 94°C for 45 s, 58°C for 45 s,

and 72°C for 60 s, were performed. Initial incubation at 94°C for 3 min, final extension at 72°C for 7 min and final soak at 4°C were included.

3.4.3. Cryptosporidium spp.

The nested PCR approach described by Xiao et al. (1999) was used to amplify a partial region of the small subunit rRNA (18S rRNA) [the primary primers F1 (5′ - TCTAGAGCTAATACATGCG - 3′) and R1 (5′ - CCCATTTCCTTCGAAACAGGA– 3′); secondary primers F2 (5′ - GGAAGGGTTGTATTTATTAGATAAAG- 3′) and R2 (5′ - AAGGAGTAAGGAACAACCTCCA - 3′)]. The primary PCR mixtures contained 2 µl of template DNA 2.5 U of Taq DNA Polymerase, 0.5×PCR buffer, 6 mM $MgCl_2$ (primary PCR) or 3 mM $MgCl_2$ (secondary PCR), 200 µM each deoxynucleoside triphosphate, 100 mM each primer and 2 µL BSA in 50 µL reaction volume. DNA of *C. serpentis* and molecular grade water were used as positive and negative controls, respectively. For both PCR steps a total number of 35 cycles, each consisting of 94°C for 45 s, 55° C for 45 s, and 72°C for 60 s, was performed. Initial incubation at 94°C for 3 min, final extension at 72°C for 7 min, and final soak at 4° C.

3.4.4. *Giardia intestinalis*

The The nested PCR approach described by Sulaiman et al. (2003a) was used to amplify a partial region of triosephosphate isomerase gene [the primary primers GIAF1 (5′-AAA TIATGCCTGCTGGTCG -3′) and GIAR1 (5′ - CAAACCTTITCCGCAAACC- 3′);the secondary primers GIAF2 (5′ -CCCTTCATCGGIGGTAACTT- 3′) and GIAR2 (5′ - GTGGCCACCACICCCGTGCC- 3′)]. The PCR mixtures contained 2 µL of template DNA, 2.5 µL of Taq DNA polymerase, 1 µL PCR buffer, 3 mM $MgCl_2$, 200 µM each deoxynucleoside triphosphate, 100 mM each primer and 2 µL BSA in 30 µL reaction volume. DNA of *G. intestinalis* assemblage F and molecular grade water were used as positive and negative controls, respectively. For both PCR steps a total number of 35 cycles, each consisting of 94°C for 45 s, 50° C for 45 s, and 72°C for 60 s, was performed. Initial incubation at 94°C for 3 min, final extension at 72°C for 7 min, and final soak at 4° C.

3.5. Gel electrophoresis

The secondary PCR products separated by electrophoresis on a 1% agarose gel containing 0.5 µg/mL ethidium bromide and visualized under UV (Thermo Fisher Scientific, Inc., USA). The agarose gel prepared by 1.2 g of agarose, added in 120 µL 1× TAE buffer to obtain 1% gel and mixed in flask laboratory glassware; the solution boiled and dissolved in a microwave oven. The glassware was placed under running water for 5 min to cool the agarose and 2 µL of ethidium bromide was added as dye for binding of the molecule to the DNA. The gel was poured into casting tray, and then the comb inserted into the gel to one end of the gel tray and left at the room temperature for approximately 40 min to coagulate. After reading agarose the tray with the gel placed in an electrophoresis casting gel tray with 1× TAE buffer, the combs were removed from the gel and 10 µL marker (ladder) was applied to the gel to the first place and the secondary PCR products was loaded to the resulting wells. The casting gel tank was connected to a source at a voltage. DNA amplicons were visualized on the gel using a UV trans-illuminator.

3.6. PCR product purification and sequencing

All secondary PCR amplicons were purified using the GenElute™ Gel Extraction Kit (Sigma-Aldrich, St. Louis, MO, USA).

Procedure description:

1. Excise the DNA fragment of interest from the agarose gel with a clean, sharp scalpel. Trim away excess gel to minimize the amount of agarose.
2. Weigh the gel slice in a tared colorless tube.
3. Add 3 gel volumes of the Gel Solubilization Solution to the gel slice (for every 100 mg of agarose gel, add 300 mL of Gel Solubilization Solution)
4. Incubate the gel mixture at 60 °C for 10 min, or until the gel slice is completely dissolved. Vortex briefly every 2–3 min during incubation to help dissolve the gel.

5. Preparation of the binding column can be completed while the agarose is being solubilized in previous step. Place the GenElute Binding Column G into one of the provided 2 mL collection tubes. Add 500 mL of the Column Preparation Solution to each binding column. Centrifuge for 1 min. Discard flowthrough liquid.
6. Once the gel slice is completely dissolved, make sure the color of the mixture is yellow - similar to fresh Gel Solubilization Solution.
7. Add 1 gel volume of 100% isopropanol and mix until homogenous.
8. Load the solubilized gel solution mixture from step 7 into the binding column that is assembled in a 2 mL collection tube. Centrifuge for 1 min after loading the column. Discard the flowthrough liquid.
9. Add 700 mL of Wash Solution to the binding column. Centrifuge for 1 min. Remove the binding column from the collection tube and discard the flow-through liquid. Place the binding column back into the collection tube and centrifuge again for 1 min without any additional wash solution to remove excess ethanol.
10. Transfer the binding column to a fresh collection tube. Add 50 mL of Elution Solution (preheated at 65°C) to the center of the membrane and incubate for 1 min. Centrifuge for 1 min.
11. Purified secondary products were sequenced in both directions using the secondary PCR primers at commercial company (Eurofins, Praha, Czech Republic).

3.7. Phylogenetic analysis

Chromatogram analyses were performed by using software Chromas Pro 2.1.4 (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 1000 replications. Phylogenetic trees were produced by MEGAX and further edited for visualization purposes with Corel DrawX7 (Corel Corporation, Ottawa, Ontario, Canada).

3.8. Real time PCR (qPCR)

The presence and amount of specific DNA of *G. intestinalis* and *E. cuniculi* was tested by qPCR and samples were processed using a Light Cycler LC 480 I (Roche, Basel, Switzerland).

Procedure description:

1. Defrost PCR reagents at room temperature. Keep probe in dark.
2. Prepare the PCR master mixture solution by adding all the PCR reagents in a 1.5 mL microcentrifuge tube as suggested in appropriate PCR target reaction (see below).
3. Mix well by vortexing briefly and centrifuge the tube for 5 s to remove any solution trapped in the tube cap.
4. Add 15 μL of the PCR master mixture prepared above to each 0.2 mL qPCR tube/plate.
5. Add 5 μL UV-treated distilled water to the tube designated as the blank negative control or add 5 μL DNA
6. Add 5 μL positive control DNA to the tube designated as positive control. The positive controls should not be handled until this step to avoid cross-contamination.
7. Mix the sample tubes by centrifuging briefly in dark.
8. Select the cycling program for the appropriate PCR reaction (see below). Start the light cycler.

3.8.1. *Encephalitozoon cuniculi*

For the qPCR, a 268 bp fragment was identified as an amplicon within the 16S RNA gene, which was amplified and detected using the qPCR protocol (Wolk et al. 2002). The following primers and probe were used F1 (5´-GTCCGTTTAGCCCTGAGA-3´), R1

(5´-ACAGCAGCCATGTTACGACT-3´) and Ence TaqMan (5´-RED640-TGGACGAAGATTGGAAGGTCTGAGTC-PHOSPHATE-3´). The qPCR mixtures contained 5 µl of template DNA, 1× FastStart Universal Probe Master (Roche), 400 mM each primer and 200 mM probe in 20 µL reaction volume. Primers and hybridization probe labelled with fluorescein reporter and Fast Start Universal Probe Master were purchased from Generi Biotech (Hradec Králové, Czech Republic). qPCRs were performed using the LightCycler® 480 System with initial denaturation step of 95°C for 10 min and amplification was performed in 50 cycles including a denaturation step at 95°C for15 s, an annealing step at 60°C for 30 s and an extension step at 72°C for 1 s. Each run included positive (DNA from spores of *E. cuniculi* genotype I) and negative (DNA water) controls consisting of unspiked specimens and diluent blanks. Results were determined to be positive, when the fluorescence signal crossed at the baseline at ≤43 cycles. The total amount of spores in 1 g of individual fecal samples was calculated using a standard curve derived from fecal samples containing from 10^1 to 10^8 spores per 1 g of feces.

3.8.2. *Giardia intestinalis*

For the qPCR diagnostics of *G. intestinalis*, a diagnostic protocol of Verweij et al. (2004) with primers targeting a 63 bp fragment of 18S RNA was applied, which was detected by a Taqman probe. The following primers and probe were used Giardia -80-F (5´-GACGGCTCAGGACAACGGTT-3´), Giardia-127-R(5´TTGCCAGCGGTGTCCG-3´) and Giardia 105 T TaqMan (FAM-CCCGCGGCGGTCCCTGCTAG-BHQ-1) (Verweij et al. 2004). All amplifications of qPCR reactions were performed in a total volume of 20 µl (500 mM of reverse primer, 500 mM of forward primer for each sample, 125 mM of TaqMan probe, 4 µl of polymerase, 8 µl of milliQ water, and 5 µl of extracted DNA). A positive control (DNA from an axenic culture of *G. intestinalis* trophozoites) and negative control (miliQ water) were added to the same 96 well plates. Samples were processed using LightCycler® LC 480 I (Roche) and under cycling conditions consisted of 10 min at 95°C, 50 × (95°C/ 15 s, 60°C/30 s,72°C/1 s). Moreover, the quantification curve for estimation of the fecal protist load was generated from a dilution series of cultured *Giardia* trophozoites between 10^{-1} and 10^5 trophozoites. Trophozoites counts from culture were calculated using a Bürker`s chamber and then serially diluted to obtain

aliquotes 10^{-1}, 10^{0}, 10^{1}, 10^{2}, 10^{3}, 10^{4} and 10^{5} cells, which were subsequently subjected to DNA extraction (Brožová 2019, 2021).

3.9. Statistics analysis

All the analyses were performed in R 4.1.3 (R Core Team 2022). A generalized linear mixed model (GLMM) was used to test effect of study area (categorical predictor with four areas) and age/sex class (categorical predictor with four classes) on probability of infection by *G. intestinalis* or *E. bieneusi* (both binary response variables – present or absent). GLMM with binomial distribution and logit link was used separately for each of the two response variables. As each study area was inhabited by several gorilla groups and we analyzed multiple samples from each group. To control for multiple sampling within each group, we included a random intercept of study group identity in the GLMMs.

4. Results

In total, 152 individual fecal samples originating from mountain gorillas were examined for presence specific DNA of *Giardia intestinalis*, *Encephalitozoon* spp., *Enterocytozoon bieneusi* and *Cryptosporidium* spp. using genus specific nested PCR and qPCR. While none of samples were positive for *G. intestinalis* using nested PCR, qPCR detected *G. intestinalis* in 20 samples in very fecal protist load ranging from 10^{-1} to 10^{1} (Table 1). Attempts to genotype *G. intestinalis* in the positive samples were repeatedly unsuccessful. *Cryptosporidium*-specific DNA was detected in one sample by nested PCR targeting the 18S rRNA gene. From this positive sample, partial sequences of 18S rDNA were sequenced and ML tree showed the presence of *C. muris* (Figure 6, Table 1). Neither PCR nor qPCR showed the presence of *Encephalitozoon*-specific DNA in any of the tested samples. *E. bieneusi*-specific DNA was detected in 22 samples (Table 1). Phylogenetic trees (ML) constructed from complete ITS sequences showed the presence of two distinct genotypes. Twenty-one sequences of 22 clustered to the previously known genotype D (GenBank accession number AF101200; Figure 7). Remaining sample was identical with *E. bieneusi* genotype CHN-F1 (Figure 7).

Table 1: The occurrence, genotypes and fecal protist load of *Cryptosporidium* spp., *Enterocytozoon bieneusi*, *Encephalitozoon* spp. and *Giardia intestinalis* in fecal samples of Virunga mountain gorillas detected by nested PCR and qPCR. Only positive samples are listed. Karisimbi-Visoke (K_V), Visoke-Sabyinyo (V_S), Sabyinyo-Muhavura (S_M), Mikeno (M).*genotype; **ct (threshold cycle) value and protist load (respectively).

			***Cryptosporidium* spp.**		***Enterocytozoon bieneusi***		***Encephalitozoon* spp.**			***Giardia intestinalis***
Sample ID	**Group**	**Area**	**PCR**	**qPCR**	**PCR***	**qPCR**	**PCR**	**qPCR**	**PCR**	**qPCR****
54982	Amahoro	V_S	-	ND	D	ND	-	-	-	**-**
54983	Amahoro	V_S	-	ND	D	ND	-	-	-	**-**
54985	Amahoro	V_S	-	ND	-	ND	-	-	-	**38, 10^{-1}**
54986	Amahoro	V_S	-	ND	D	ND	-	-	-	**-**
54987	Amahoro	V_S	-	ND	D	ND	-	-	-	**37, 10^{-1}**
54988	Amahoro	V_S	-	ND	-	ND	-	-	-	**37, 10^{-1}**
54550	Bageni	M	-	ND	-	ND	-	-	-	**38, 10^{1}**
54555	Bageni	M	-	ND	D	ND	-	-	-	**-**
54559	Bageni	M	-	ND	D	ND	-	-	-	**38, 10^{1}**
54560	Bageni	M	-	ND	-	ND	-	-	-	**37, 10^{1}**
54562	Bageni	M	-	ND	D	ND	-	-	-	**-**
54564	Bageni	M	-	ND	D	ND	-	-	-	**-**

54554	Bageni	M	-	ND	-	ND	-	-	-	**40, 10^{-1}**
54963	Hirwa	S_M	-	ND	D	ND	-	-	-	**-**
54964	Hirwa	S_M	-	ND	D	ND	-	-	-	**-**
54966	Hirwa	S_M	-	ND	D	ND	-	-	-	**-**
54967	Hirwa	S_M	-	ND	D	ND	-	-	-	**-**
54840	Hirwa	S_M	-	ND	-	ND	-	-	-	**37, 10^{1}**
54842	Hirwa	S_M	-	ND	-	ND	-	-	-	**39, 10^{1}**
55347	Humba	M	-	ND	-	ND	-	-	-	**39, 10^{1}**
54848	Igisha	K_V	-	ND	-	ND	-	-	-	**38, 10^{1}**
54849	Igisha	K_V	-	ND	D	ND	-	-	-	**-**
54853	Igisha	K_V	-	ND	D	ND	-	-	-	**-**
54854	Igisha	K_V	-	ND	-	ND	-	-	-	**39, 10^{-1}**
54856	Igisha	K_V	-	ND	-	ND	-	-	-	**39, 10^{1}**
54961	Iyambere	K_V	-	ND	-	ND	-	-	-	**38, 10^{1}**
54565	Kabirizi	M	-	ND	-	ND	-	-	-	**37, 10^{1}**
54571	Kabirizi	M	-	ND	D	ND	-	-	-	**-**
54974	Kwitonda	S_M	-	ND	D	ND	-	-	-	**-**
54976	Kwitonda	S_M	-	ND	D	ND	-	-	-	**-**
54977	Kwitonda	S_M	-	ND	-	ND	-	-	-	**38, 10^{-1}**
55348	Lulengo	M	-	ND	-	ND	-	-	-	**40, 10^{1}**
54579	Lulengo	M	-	ND	-	ND	-	-	-	**48, 10^{1}**
55020	Musirikare	K_V	-	ND	D	ND	-	-	-	**-**

54875	Musirikare	K_V	-	ND	CHN-F1	ND	-	-	-	-
54882	Musirikare	K_V	-	ND	D	ND	-	-	-	**40, 10^{-1}**
54992	Sabyinyo	V_S	-	ND	D	ND	-	-	-	-
54994	Sabyinyo	V_S	*C. muris*	ND	-	ND	-	-	-	-
54870	Susa	K_V	-	ND	D	ND	-	-	-	**39, 10^{1}**

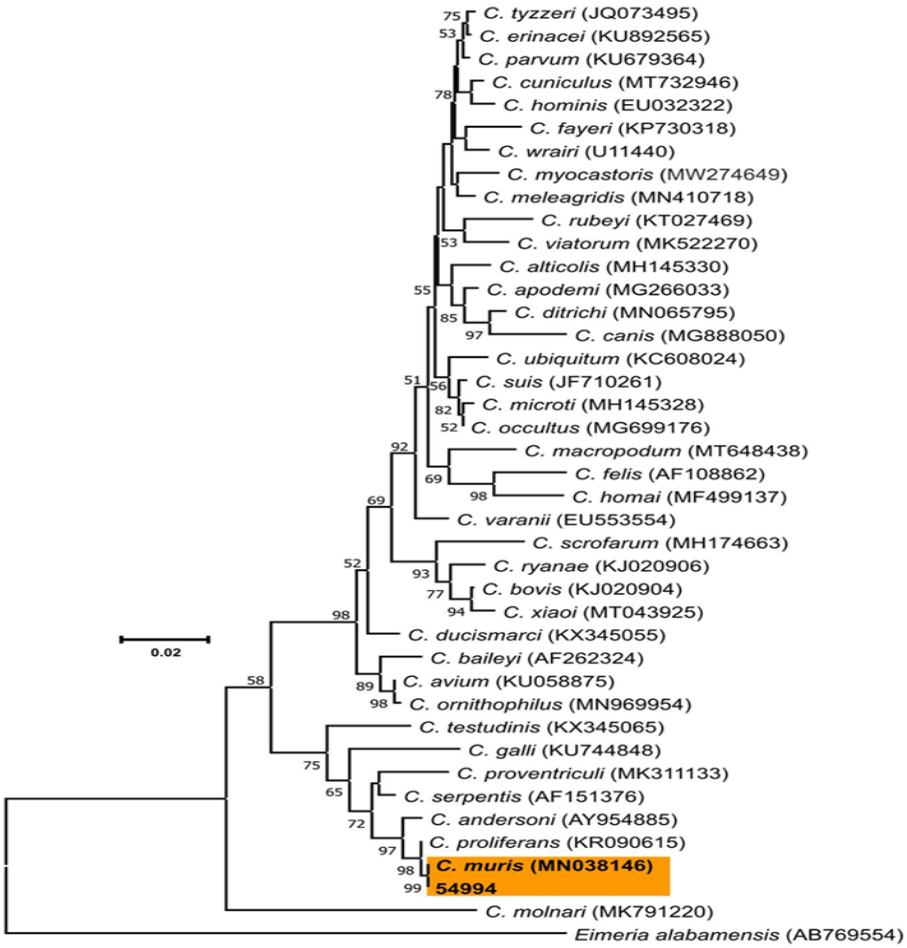

Figure 6: Phylogenetic relationships among *Cryptosporidium muris* detected in the present study (highlighted) and other *Cryptosporidium* spp. deposited in GenBank as inferred by Maximum Likelihood (ML) analyses (General Time Reversible model with a gamma distribution) of partial region of the small subunit rRNA (18S rRNA). Percentage support (>50%) from 1000 pseudo-replicates from ML are indicated next to supported node. The tree 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 18S rDNA sequence of *Eimeria alabamensis*. The sequences obtained in this study is identified by the isolation number (54994). The GenBank Accession number is in parenthesis.

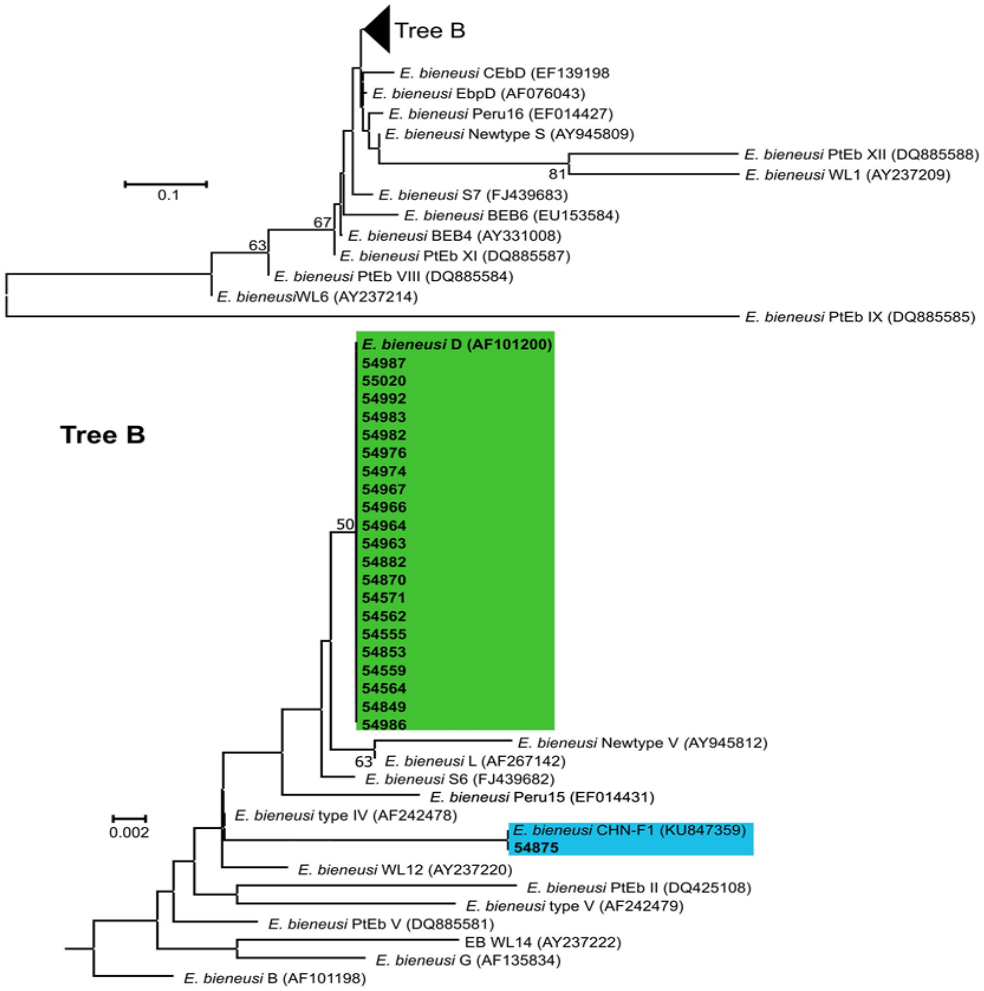

Figure 7: **Phylogenetic relationships among genotypes of *Enterocytozoon bieneusi* detected in the present study (highlighted) and other isolates deposited in GenBank as inferred by Maximum Likelihood (ML) analyses (General Time Reversible model with a gamma distribution) of the internal transcribed spacer 1 (ITS).** Percentage support (>50%) from 1000 pseudoreplicates from ML are indicated next to 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* PtEb IX genotype. Sequences obtained in this study are identified by isolate number (e.g. 54987). The GenBank Accession number is in parenthesis.

G. intestinalis infection probabilities did not differ among study areas (GLMM; chi-square = 2.8, df = 3, p = 0.43; Table 2A) or age/sex classes (chi-square = 2.9, df = 3, p = 0.41; Table 2A). Detailed pairwise comparisons of estimated marginal means from GLMM did not detect significant differences among four study areas (all p > 0.40; Table 3A) or age/sex classes (all p > 0.40; Table 3A). Similarly, *E. bieneusi* infection probabilities did not differ among study areas (GLMM; chi-square = 2.7, df = 3, p = 0.44; Table 2B) or age/sex classes (chi-square = 4.1, df = 3, p = 0.25; Table 2B). Detailed pairwise comparisons of estimated marginal means from GLMM did not detect significant differences among four study areas (all p > 0.40; Table 3B) or age/sex classes (all p > 0.30; Table 3B).

Table 2: ***Giardia intestinalis*** **(A) and** ***Enterocytozoon bieneusi*** **(B) prevalence at four study areas and four age/sex classes within Virunga Massif.** Karisimbi-Visoke (K_V), Visoke-Sabyinyo (V_S), Sabyinyo-Muhavura (S_M), Mikeno (M).

(A)

Predictor	Category	N	Mean prevalence (%)	Lower CI	Upper CI
Study area	K_V	59	10.2	3.8	20.8
	M	39	20.5	9.3	36.5
	S_M	32	9.4	2.0	25.0
	V_S	22	13.6	2.9	34.9
Age/sex class	Infants	29	6.9	0.8	22.8
	Juveniles/Subadults	26	11.5	2.5	30.2
	Adults	69	13.0	6.1	23.3
	Silverbacks	28	21.4	8.3	41.0

(B)

Predictor	Category	N	Mean prevalence (%)	Lower CI	Upper CI
Study area	K_V	59	10.2	3.8	20.8
	M	39	12.8	4.3	27.4
	S_M	32	18.8	7.2	36.4
	V_S	22	22.7	7.8	45.4
Age/sex class	Infants	29	6.9	0.8	22.8
	Juvenile/Subadults	26	19.2	6.6	39.4
	Adults	69	18.8	10.4	30.1
	Silverbacks	28	7.1	0.9	23.5

Table 3: **Pairwise comparisons probability of infection by *Giardia intestinalis* (A) and *Enterocytozoon bieneusi* (B) among four study areas and age/sex classes in Virunga Massif.** Values are marginal means estimated by generalized linear mixed models. Karisimbi-Visoke (K_V), Visoke-Sabyinyo (V_S), Sabyinyo-Muhavura (S_M), Mikeno (M). juv/sub – juveniles/subadults.

(A)

Predictor	Contrast	Odds ratio±SE	z ratio	P - value
Area	K_V : M	0.40±0.24	1.51	0.43
	K_V : S_M	0.97±0.75	0.04	1.00
	K_V : V_S	0.60±0.47	0.65	0.91
	M : S_M	2.40±1.77	1.18	0.64

	M : V_S	1.49±1.13	0.53	0.95
	S_M : V_S	0.62±0.56	0.53	0.95
Age/sex	Adults : Infants	1.91±.58	0.78	0.86
	Adults : Juv/Sub	1.14±0.84	0.18	0.99
	Adults : Silverbacks	0.49±0.29	1.18	0.64
	Infants : Juv/sub	0.60±0.59	0.52	0.95
	Infants : Silverbacks	0.25±0.23	1.53	0.42
	Juv/Sub : Silverbacks	0.43±0.34	1.06	0.71

(B)

Predictor	Contrast	Odds ratio±SE	z ratio	P - value
Area contrast	K_V : M	0.88±0.62	0.18	0.99
	K_V : S_M	0.48±0.33	1.06	0.72
	K_V : V_S	0.33±0.25	1.46	0.46
	M : S_M	0.54±0.41	0.82	0.85
	M : V_S	0.38±0.31	1.82	0.64
	S_M : V_S	0.70±0.55	0.46	0.97
Age/sex contrast	Adults : Infants	3.86±3.16	1.65	0.35
	Adults : Juv/Sub	1.10±0.69	0.15	0.99
	Adults : Silverbacks	2.91±2.37	1.31	0.55
	Infants : Juv/Sub	0.28±0.26	1.37	0.52
	Infants : Silverbacks	0.75±0.81	0.26	0.99
	Juv/sub : Silverbacks	2.65±2.48	1.05	0.72

5. Discussion

Using molecular diagnostics I detected microsporidia *Enterocytozoon bieneusi* in 14 % prevalence, *Cryptosporidium muris* in prevalence of less than 1% of and *Giardia intestinalis* in prevalence 13% in fecal samples from multiple mountain gorilla groups inhabiting Virunga Massif. Overall, the prevalence of those opportunistic protists in Virunga mountain gorillas was comparable to the previous studies of great apes (Sak et al. 2013, 2014; Mynářová et al. 2016). The analyses of the ITS sequences revealed two genotypes of *E. bieneusi*, more common genotype D present in several gorilla groups and only in one sample I identified genotype CHN-F1. *E. bieneusi* genotype D were identical to e.g. isolates form humans (*Homo sapiens*), Korean water deer (*Hydropotes inermis argyropus*), Raccoon dogs (*Nyctereutes procyonoides*) or rhesus macaque (*Macaca mulatta*) (Chalifoux et al. 2000; Sulaiman et al. 2003b; Li et al. 2016; Amer et al. 2019).

Found genotype *E. bieneusi* CHN-F1 was identical to isolates from Raccoon dogs (*Nyctereutes procyonoides)* (Zhao et al. 2015; Xue et al. 2016) and Blue fox (*Alopex lagopus*) (Zhao et al. 2015). The sequence of *C. muris* was identical to e.g. isolates from Brown Rat (*Rattus norvegicus)* (Iseki 1986; Thompson & Ash 2016), HIV positive human (*H. sapiens*) (Guyot et al. 2001; Gatei et al. 2002; Palmer et al. 2003), Bactrian camel (*Camelus bactrianus*) (Kváč et al. 2008; Wang et al. 2021), Sumatran orangutan (*P.o abelii*) (Dubey et al. 2002; Mynářová et al. 2016).

My results were only partially consistent with the previous study of (Sak et al. 2014) and indicating lower protist infections in 2018 in comparison 2007 and showing the shifts in studied protist communities in Virunga gorillas. Sak et al. (2014) detected multiple genotypes of *E. bieneusi* (18%), namely EbpA, C, D, gorilla 2 and five novel genotypes gorilla 4–8 and *E. cuniculi* with genotype I and II (11%), *C.muris* (less than 1%) and *C. meleagridis* (less than 1%) in gorillas in the Volcanoes National Park, Rwanda. Thus, when I observed a significant decrease in diversity of microsporidia infections in Virunga gorilla population in 2018 samples in comparison to samples collected in 2007 (Sak et al. 2014). I found no *Encephalitozoon* infections. For *E. bieneusi* only genotype D was found in both studies (only in Kwitonda gorilla group in both 2007 and 2018) and only one *Cryptosporidium* genotype was detected, while Sak et al. (2014) found two in 2007 samples. I speculate that it is possible that *E. bieneusi* genotype D

might gradually replace other genotypes found previously and became dominant in mountain gorillas across Virunga Massif. Similar situation was partially observed in captive apes, the results indicated that in the captive apes, the genotypes common in the wild were replaced by those from other hosts (Sak et al. 2011a). In comparison to 2007 (Sak et al. 2014), I found significant increase in *G. intestinalis* infections, however, this was apparently caused by implementation of more sensitive method in my study, specifically qPCR as shown before (see e.g. Capewell et al. 2020; Belkessa et al. 2021).

To my knowledge there is no similar study in any host focusing on long-term temporal changes in *Giardia, Cryptosporidium* and/or microsporidia infections with exception of Sak et al. (2013), who studied those protists in habituated western lowland gorilla for three years and found no changes in microsporidia infections among years. It is well known that microsporidia are ubiquitous in the environment, and therefore many gorillas are probably infected with microsporidia during their lifetime (Sak et al. 2011a). However, the quantity and frequency of spores shed by immunocompetent individuals are very low and sporadic and repeated sampling of the same individuals is desirable (Sak et al. 2011b). In order to cover whole Virunga Massif, we could not achieve repeated sampling of the same individuals and had to implement nest sampling similar to Sak et al. (2014). I suggest repeated and ideally long-term sampling of the same individuals is needed to clarify diversity of microsporidia infections in endangered mountain gorillas.

Both found *E. bieneusi* genotypes D and CHN-F1 belong to group 1 (Thellier & Breton 2008; Zhao et al. 2015), which are microsporidia with zoonotic potential and of public health significance (Thellier & Breton 2008). Many mammalian hosts can be infected with *E. bieneusi* genotype D including domestic and wild animals and humans with AIDS (Sak et al. 2008; Li et al. 2011; Santín & Fayer 2011a; Mori et al. 2013). *E. bieneusi* genotype D was detected also in other great apes, namely in western lowland gorillas (Sak et al. 2013), both wild and semi- captive Sumatran orangutans (*P. abelii* and *P. pygmaeus*) (Mynářová et al. 2016) and the chimpanzees (*Pan troglodytes*) from zoological gardens and sanctuaries (Sak et al. 2011d). The second found genotype CHN-F1 have been identified in carnivores in China (Zhao et al. 2015; Xu et al. 2016), thus its presence in mountain gorillas is quite surprising. This is a first case of CHN-F1 detection in other than carnivore host. Unfortunately, the potential role of humans and/or domestic and even wild animals as a source of *E. bieneusi* genotype D or sympatric carnivores (or

even possibly dogs roaming around the Park) as source for the infection with *E. bieneusi* genotype CHN-F1 for Virunga mountain gorillas remains speculative, since no current data about microsporidia in other animals and humans sharing the habitat with gorillas have been available yet. My study thus calls for more research on protist infections in various sympatric animals and humans co-habiting same ecosystem.

The lack of *Encephalitozoon* infections in gorillas in my study, even with qPCR approach implemented, was surprising, because several studies commonly revealed *E. cuniculi* in great apes including Virunga gorilla population, (Sak et al. 2013, 2014; Mynářová et al. 2016), which led to an assumption that *Encephalitozoon* infections might be present in all ape populations, but see Cibot et al. (2021). *E. cuniculi* genotypes are not host specific and have been identified e.g. in rodents, lagomorphs, carnivores, human and nonhuman primates, and birds (Canning & Hollister 1987; Reetz 1993; Kašičková et al. 2009). For example, in Central African Republic, *E. cuniculi* genotypes I and II were detected not only in western lowland gorillas, but also in sympatric ruminants (*Syncerus caffer*, *Cephalophus callipygus*, *C. dorsalis*) (Sak et al. 2013). I have carefully assessed possible reasons for failure of both nested and qPCR, mostly the risk of PCR inhibition, but as other microsporidia (*E. bieneusi*) were detected, we excluded this reason. As mentioned above, microsporidia spores are intermittently shed into the environment, but with sample size n=152 we believe we should be able to detect infections at least in some individuals.

Detection of just one case of *C. muris* infection in my study resembles results of Sak et al. (2014), although this infection was found in different group in comparison to Sak et al. (2014). *Cryptosporidium muris* is reported to infect mammals and domestic animals such as goat, cattle and pets (Kodádková et al. 2010; Ghazy et al. 2015; Thompson & Ash 2016; Kváč et al. 2017; Zahedi et al. 2016) and also in humans (Chappell et al. 2015). Different *Cryptosporidium* species were found in gorillas previously, namely *C. meleagridis* in the Virunga population previously (Sak et al. 2014), *C. parvum* (Graczyk et al. 2001) and *C. bovis* (Sak et al. 2013). Most of the studies on great apes implementing molecular tools report low prevalence of *Cryptosporidium* infections in great apes (e.g. Graczyk et al. 2001; Gillespie et al. 2009; Sak et al. 2013, 2014; Butel et al. 2015; Kalema-Zikusoka et al. 2018), even in the apes positive with Simian immunodeficiency virus (Butel et al. 2015). The source of cryptosporidial

infection in Virunga mountain gorilla reported in this and previous study (Sak et al. 2014) remains unknown, however, it is possible that under certain conditions this host specific cryptosporidia can be transmitted among species (Robinson et al. 2008; Kváč et al. 2009). *C. muris* has been previously reported to be human pathogenic in both immunocompetent and immunodecificient human patients (Guyot et al. 2001). As this pathogen can cause serious diarrhea in symptomatic people with possibly fatal impacts in immunodeficient individuals (Kváč et al. 2009), I suggest to keep careful monitoring of *Cryptosporidium* infections in Virunga gorillas.

In my study, I showed that for successful detection of low infections of *G. intestinalis* in wild host populations, qPCR is more optimal diagnostic approach than nested PCR. Real time PCR (qPCR) is highly sensitive tool compared to conventional PCR, which is used for assemblage determination, but detects infection intensities only from 10^4 (Brožová 2019, 2021). In general, a range of studies used different methods for *Giardia* diagnostics in gorillas (e.g. coproscopy, fecal immunoassays, conventional PCR), which differ in their sensitivities. Thus, I did not attempt to compare found prevalence of *G. intestinalis* in Virunga mountain gorillas with other studies, which did not employ qPCR for *Giardia* screening in gorillas. Only Menu et al. (2021) used qPCR for *Giardia* screening in western lowland gorillas and found high prevalence *G. intestinalis* (26%) compared to studies using nested PCR approach (e.g. Hogan et al. 2014; Sak et al. 2014). The identification of *G. intestinalis* assemblages from the samples of Virunga gorillas in my study failed. This was caused by low intensities of *G. intestinalis* in the examined samples as detected by qPCR (10^{-1} to 10^1) (Brožová 2021). *G. intestinalis* is well-known zoonotic protist that infects a range of animals including non-human primates, livestock and human and up to date it has been sub-classified into eight genetic assemblages (designated A–H) (Heyworth 2016; Thompson & Ash 2016). Without knowledge of assemblage(s) infecting Virunga mountain gorillas, it is impossible to consider sources of infections. As mentioned above for the other protists, it is warranted to screen also other sympatric wild and domestic animals and humans for *Giardia* infections and identify found assemblages to properly reconstruct possible transmission patterns among sympatric hosts (e.g. Graczyk et al. 2002b; Sak et al. 2013; Cibot et al. 2021). Hogan et al. (2014) detected *Giardia* assemblage B of Virunga gorilla samples and assemblage E in the cattle samples, while in Bwindi gorillas assemblage A

was identified in mountain gorillas, humans and cattle (Graczyk et al. 2002b). Sak et al. (2013) found assemblage A (subgroup A II) in both in humans and lowland gorillas, which suggested that human-gorilla transmission, while a goat was infected by assemblage E. These studies used nested PCR for *Giardia* detection. I implemented a sensitive approach that revealed very low infection intensities of *G. intestinalis*, but assemblage identification was challenging. Therefore, it is necessary to introduce sensitive methodical approach that will enable not only to detect the presence of *G. intestinalis* in wild host, like mountain gorillas, which often harbor infections in low intensities, but will also allow assemblage identification. Development of such approach is under the process in the laboratories at the Institute of Parasitology, Biology Centre, Czech Academy of Sciences.

Due to low number of positive samples for *Cryptosporidium* spp., I could not analyze geographical differences in *Cryptosporidium* infections. Thus, I explored differences among areas in Virunga Massif as defined by Petrželková et al. (2021) by position of the volcanos (a proxy for the occurrence of gastrointestinal disease, vegetation types, gorilla subpopulation growth and associated changes in social structure) only for microsporidia and *Giardia* infections. I found no differences in the occurrence of microsporidia and *Giardia* infections among K_V, V_S, S_M and M areas. Petrzelkova et al (2021) found higher strongylid egg counts in K_V and V_S areas in comparison to S_M and M area. The analyses of the composition of strongylid communities in the Virunga Massif using high throughput sequencing approach revealed dominance of pathogenic gastric species in gorillas inhabiting the K_V area (where most of the gastritis cases were observed), while in the V_S the communities were dominated by intestinal, probably non-pathogenic species (Pafco et al., unpublished data). Interestingly, gorillas in M and S_M harbored those gastric strongylids too, but when combined with the egg counts (proxy for the intensities of infection), their infections were extremely low in comparison to gorillas inhabiting V_S area (Pafco et al., unpublished data). Based on the observed strongylid patterns, I expected higher occurrence of studied opportunistic pathogens. Moreover, Sak et al. (2014) observed increased prevalence of microsporidia infections in previously so-called “research groups” of mountain gorillas in comparsion to “tourist groups” in Volcanos NP. The “research groups” range in K_V area, while the “tourist groups” in V_S area, indicating some patterns which might be consistent with

those observed in strongylids (Petrzelkova et al., 2012). In the last years the gorilla subpopulation in the in K_V areas (aka Karisoke research area, area between Mount Karisimbi and Mount Visoke) increased in size by almost 50%, group density increased and gorilla annual home ranges groups decreased (Caillaud et al. 2014, 2020). Subsequently, changes in the social behavior were observed, e.g. a fivefold increase in the rate of infanticide, feeding competition, male-male aggression and stress (Caillaud et al. 2020). Observed intergroup encounters were significant source of stress as observed by increased fecal glucocorticoid metabolite levels up to almost nine times, and this could influence susceptibility to pathogens (Eckardt et al. 2016, 2019; Petrželková et al. 2022). However, based on my results, I conclude that opposite to strongylids, no increase in the occurrence of microsporidia and *Giardia* infections in the K_V area was observed and probably studied protists were not impacted by the changes in mountain gorilla population dynamics in Virunga Masiff.

I also did not observe any differences in the occurrence of studied protists among defined sex/age categories. There are inconsistencies in the impact of age on *Giardia* infection, with some studies reporting no differences among age categories (e.g. horses: (Santín et al. 2013), or higher prevalence in subadult versus adult animals (livestock: (Johnston et al. 2010), while other studies reported higher prevalence in adult humans (Johnston et al. 2010; Ferguson et al. 2020; Brožová 2021), but some in children in comparison to other categories (Oyerinde et al. 1977). In non-human primates, no age differences was reported in black and gold howler monkeys (*Alouatta caraya*) inhabiting fragmented forests (Alegre et al. 2021). To my knowledge there are no studies exploring age differences in microsporidia infections.

6. Conclusions

In this study, I explored the microsporidia, *Giardia* and *Cryptosporidium* infections across the Virunga mountain gorilla population. Using non-invasive fecal sampling from night gorilla nests and molecular diagnostics, I found out that *Enterocytozoon bieneusi* (genotype D) (14%) and *G. intestinalis* (13%) were the most prevalent infections. In addition, I detected a single infection of *C. muris* and a single infection of *E. bieneusi* (genotype CHN-F1) (both less than 1% prevalence). The qPCR was a more effective technique in comparison to nested PCR to successfully detect low infections of *Giardia* in gorilla fecal samples, but unsuitable for assemblage identification. I revealed temporal changes in studied protist infections in comparison to results of Sak et al. (2014). I found no spatial and sex/age differences in studied protists. I conclude that currently microsporidia, *Giardia* or *Cryptosporidium* infections probably do not pose a threat to the Virunga mountain gorilla population. However, to identify the sources of found infections I suggest systematic pathogen monitoring of sympatric wildlife and domestic animals and humans living in the proximity of the park. I also suggest to continue monitoring of these protist infections in gorillas in future using repeated individual sampling. Parasite infection in wild non-human primate populations rarely cause host mortality and they are rather 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), call for the strategic conservation plans, which should include regular monitoring of parasite infections and also consider the spread of parasites via environmental contamination among gorillas to ensure sustainable protection and survival of the endangered Virunga mountain gorillas.

7. References

Ahmed DA, Rabbo MA, Jamjoom M, Salem HS, Ghieth MA. 2019. Giardiasis Pattern among Different Age Categories: Childhood Assemblage B Proclaim Endemicity. Iranian Journal of Parasitology **14**:614–622. DOI: 10.18502/ijpa.v14i4.2103.

Alegre RE, Gennuso MS, Milano F, Kowalewski M. 2021. Relationship between age-sex classes and prevalence of Giardia spp . and Blastocistys spp . in black and gold howler monkeys inhabiting fragmented forests. Therya **12**:563–569. DOI: 10.12933/therya-21-1156.

Amer S, Kim S, Han JI, Na KJ. 2019. Prevalence and genotypes of *Enterocytozoon bieneusi* in wildlife in Korea : a public health concern. Parasites & Vectors **12**:160

Appelbee AJ, Thompson RCA, Olson ME. 2005. *Giardia* and *Cryptosporidium* in mammalian wildlife – current status and future needs. Trends in Parasitology **21**:370–373. DOI: 10.1016/j.pt.2005.06.004.

Belkessa S, Thomas-Lopez D, Houali K, Ghalmi F, Stensvold CR. 2021. Molecular Characterization of Giardia Duodenalis in Children and Adults Sampled in Algeria. Microorganisms **54**:1–11. DOI: 10.3390/ microorganisms9010054.

Berrilli F, Prisco C, Friedrich KG, Cerbo P Di, Cave D Di, Liberato C De. 2011. Giardia duodenalis assemblages and Entamoeba species infecting non-human primates in an Italian zoological garden : zoonotic potential and management traits. Parasites & Vectors **4**:199. DOI: 10.1186/1756-3305-4-199.

Bizawu K, Gomes MF. 2016. Oil exploitation at Virunga Park as a threat to the Environment and to Endangered Animal Species. Veredas do Direito, Belo Horizonte **13**:11–29.

Bojko J, Reinke AW, Stentiford GD, Rogers MSJ, Bass D, Williams B. 2022. Microsporidia : a new taxonomic , evolutionary , and ecological synthesis. Trends in Parasitology **38**:642–659. DOI: 10.1016/j.pt.2022.05.007.

Bradbury RS, Pafčo B, Nosková E, Hasegawa H. 2021. Strongyloides genotyping : a review of methods and application in public health and population genetics. International Journal for Parasitology **51**:1153–1166. DOI:

10.1016/J.IJPARA.2021.10.001.

Brožová K. 2019. Studium prevalence G. intestinalis [Study of prevalence G. intestinalis. Bc. Thesis, in Czech]. Faculty of Science, University of South Bohemia, České Budějovice, Czech Republic.

Brožová K. 2021. Průzkum výskytu oportunního prvoka , Giardia intestinalis , u zdravých lidí a jejich zvířat v ČR s použitím qPCR. Diplomová práce. Jihočeská univerzita v Českých Budějovicích, České Budějovice, Czech Republic.

Buckholt MA, Lee JH, Tzipori S. 2002. Prevalence of Enterocytozoon bieneusi in swine: An 18-month survey at a slaughterhouse in Massachusetts. Applied and Environmental Microbiology **68**:2595–2599. DOI: 10.1128/AEM.68.5.2595-2599.2002.

Butel C, Mundeke SA, Drakulovski P, Krasteva D, Ngole EM, Mallié M, Delaporte E, Peeters M, Locatelli S. 2015. Assessment of Infections with Microsporidia and Cryptosporidium spp. in Fecal Samples from Wild Primate Populations from Cameroon and Democratic Republic of Congo. International Journal of Primatology **36**:227–243.

Caillaud D, Eckardt W, Vecellio V, Ndagijimana F, Mucyo JP, Hirwa J., Stoinski T. 2020. Violent encounters between social units hinder the growth of a high-density mountain gorilla population. Science Advances **6**:1–10.

Caillaud D, Ndagijimana F, Giarrusso AJ, Vecellio V, Stoinski TS. 2014. Mountain gorilla ranging patterns: Influence of group size and group dynamics. American Journal of Primatology **76**:730–746. DOI: 10.1002/AJP.22265.

Cali A, Becnel JJ, Takvorian PM. 2017. Microsporidia. Pages 1559–1618 in Archibald J, Simpson A, Slamovits C, editors. Handbook of the Protists: Second Edition.Springer International Publishing. DOI: 10.1007/978-3-319-28149-0.

Canning E, Hollister W. 1987. Microsporidia of Mammals- Widespread Pathogens or Opportunistic Curiosities ? Parasitology Today **3**:267–273.

Capewell P, Krumrie S, Katzer F, Alexander CL, Weir W. 2020. Molecular Epidemiology of Giardia Infections in the Genomic Era. Trends in Parasitology **37**:142–153. DOI:

10.1016/j.pt.2020.09.013.

Carreno RA, Martin S, Barta JR. 1999. Cryptosporidium is more closely related to the gregarines than to coccidia as shown by phylogenetic analysis of apicomplexan parasites inferred using small-subunit ribosomal RNA gene sequences. Parasitology Research **85**:899–904.

Cavalier-Smith T. 2014. Gregarine site-heterogeneous 18S rDNA trees , revision of gregarine higher classification , and the evolutionary diversification of Sporozoa. European Journal of Protistology **50**:472–495. Elsevier GmbH. DOI: 10.1016/j.ejop.2014.07.002.

Červená B, Brant SV, Fairet E, Shirley MH, Petrželková KJ, Modrý D. 2016. Schistosoma mansoni in Gabon : Emerging or Ignored ? American Journal of Tropical Medicine and Hygiene **95**:849–851.

Chappell CL, Okhuysen PC, Langer-curry RC, Lupo PJ, Widmer G, Tzipori S. 2015. Cryptosporidium muris : Infectivity and Illness in Healthy Adult Volunteers. American Society of Tropical Medicine and Hygiene **92**:50–55.

Choy SH, Al-Mekhlafi HM, Mahdy MAK, Nasr NN, Sulaiman M, Lim YAL, Surin J. 2014. Prevalence and associated risk factors of Giardia infection among indigenous communities in rural Malaysia. Scientific Reports **4**:1–9. DOI: 10.1038/srep06909.

Cibot M, McLennan MR, Kváč M, Sak B, Asiimwe C, Petrželková K. 2021. Sparse evidence for Giardia intestinalis, Cryptosporidium spp. And microsporidia infections in humans, domesticated animals and wild nonhuman primates sharing a farm-forest mosaic landscape in Western Uganda. Pathogens **10**:1–12.

Costello L, Franklin R. 2009. Integration of Genetic, Proteomic, and Metabolic Approaches in Tumor Cell Metabolism. Page (in Costello L, Singh K E, editor) Mitochondria and Cancer. Springer. New York.

Daszak P, Cunningham AA, Hyatt AD. 2000. Emerging Infectious Diseases of Wildlife — Threats to Biodiversity and Human Health. Wildlife Ecology **287**:443–449.

De Bosschere H, Wang Z, Orlandi PA. 2007. First Diagnosis of Encephalitozoon intestinalis and E . Hellem in a European Brown Hare (Lepus europaeus) with

Kidney Lesions **54**:131–134.

Didier ES, Stovall ME, Green LC, Brindley PJ, Sestak K, Didier PJ. 2004. Epidemiology of microsporidiosis: Sources and modes of transmission. Veterinary Parasitology **126**:145–166.

Direkthilfe R, Berggorilla. 2007. Gorilla. Gorilla journal. **34**

Dixon BR. 2021. Research in Veterinary Science Giardia duodenalis in humans and animals – Transmission and disease. Research in Veterinary Science **135**:283–289. Elsevier Ltd. DOI: 10.1016/j.rvsc.2020.09.034.

Doležalová J. 2018. Anoplocephalid tapeworms. Pages 126-129 in Modrý D, Pafčo B, Petrželková, KJ, Hasegawa H, editors. Parasites of Apes, An Atlas of Coproscopic Diagnostics. Edition Chimaira F am .

Dubey JP, Markovits JE, Killary KA. 2002. Cryptosporidium muris-like Infection in Stomach of Cynomolgus Monkeys (Macaca fascicularis). Veterinary pathology **39**: 363-371

Eckardt W, Fawcett K, Fletcher AW. 2016. Weaned age variation in the Virunga mountain gorillas (Gorilla beringei beringei): influential factors Weaned age variation in the Virunga mountain gorillas (< Gorilla beringei beringei): influential factors. Behavioral Ecology and Sociobiology **70**:493–507.

Eckardt W, Stoinski TS, Rosenbaum S, Santymire R. 2019. Social and ecological factors alter stress physiology of Virunga mountain gorillas (Gorilla beringei beringei). Ecology and Evolution:1–12. DOI: 10.1002%2Fece3.5115.

FAO. 2014. Multicriteria-Based Ranking for Risk Management of Food-Borne Parasites. Microbiological Risk Assessment Series (MRA) 23.

Feng Y, Yang W, Ryan U, Zhang L, Kváč M, Koudela B, Modrý D, Li N, Fayer R, Xiao L. 2011. Development of a multilocus sequence tool for typing Cryptosporidium muris and Cryptosporidium andersoni. Journal of Clinical Microbiology **49**:34–41.

Ferguson LC, Palmer AS, Alexander CL. 2020. An update on the incidence of human giardiasis in Scotland , 2011 – 2018. Parasites & Vectors:1–7. BioMed Central. DOI: 10.1186/s13071-020-04160-9.

Freeman AS, Kinsella JM, Cipolletta C, Deem SL, Karesh WB. 2004. Endoparasites of Western Lowland Gorillas (Gorilla gorilla gorilla) at Bai Hokou , Central African Republic. Journal of Wildlife Diseases **40**:775–781.

Ganas J, Robbins MM. 2005. Ranging behavior of the mountain gorillas (Gorilla beringei beringei) in Bwindi Impenetrable National Park, Uganda : a test of the ecological constraints model. Behavioral Ecology and Sociobiology **58**:277–288.

Gatei W, Ashford RW, Beeching NJ, Kang'ethe Kamwati S, Greensill J, Anthony Hart C. 2002. Cryptosporidium muris infection in an HIV-infected adult, Kenya. Emerging Infectious Diseases **8**:204–206.

Gates G, Jenkins M, Osborne N. 2020. How Africa's Mountain Gorillas Staged a Comeback. Smithsonian Magazine: Planet Positive. Washington, DC. Available from https://www.smithsonianmag.com/science-nature/africas-mountain-gorillas-staged-comeback-180974363 (accessed August 2022).

Ghazy A, Abdel-shafy S, Shaapan R. 2015. Cryptosporidiosis in Animals and Man: 1. Taxonomic Classification, Life Cycle, Epidemiology and Zoonotic Importance. Asian Journal of Epidemiology **8**:48–63.

Gilardi K, Nziza J, Ssebide B, Kambale E, Muvunyi R, Aruho R, Shalukoma C, Seguya A, Masozera AB. 2021. Endangered mountain gorillas and COVID-19: One health lessons for prevention and preparedness during a global pandemic. American Journal of Primatology **84**:4–5.

Gillespie TR, Morgan D, Deutsch JC, Kuhlenschmidt MS, Salzer JS, Cameron K, Reed T, Sanz C. 2009. A legacy of low-impact logging does not elevate prevalence of potentially pathogenic protozoa in free-ranging gorillas and chimpanzees in the Republic of Congo: Logging and parasitism in African Apes. EcoHealth **6**:557–564.

Gillespie TR, Nunn CL, Leendertz FH. 2008. Integrative approaches to the study of primate infectious disease: Implications for biodiversity conservation and global health. American Journal of Physical Anthropology **137**:53–69.

Gladys KZ, Jessica MR, Mark TF. 2004. Intestinal parasites and bacteria of mountain gorillas (Gorilla beringei beringei) in Bwindi Impenetrable. Cornell University **46**:59–63.

Graczyk TK, Bosco-Nizeyi J, Da Silva AJ, Moura IN, Pieniazek NJ, Cranfield MR, Lindquist AH. 2002a. A single genotype of Encephalitozoon intestinalis infects free-ranging gorillas and people sharing their habitats in Uganda. Parasitology Research **88**:926–931.

Graczyk TK, Bosco-Nizeyi J, Ssebide B, Thompson RCA, Read C, Cranfield MR. 2002b. Anthropozoonotic Giardia duodenalis genotype (Assemblage) A infections in habitats of free-ranging human-habituated gorillas, Uganda. Journal of Parasitology **88**:905–909.

Graczyk TK, DaSilva AJ, Cranfield MR, Nizeyi JB, Kalema GRNN, Pieniazek NJ. 2001. Cryptosporidium parvum Genotype 2 infections, in free-ranging mountain gorillas (Gorilla gorilla beringei) of the Bwindi Impenetrable National Park, Uganda. Parasitology Research **87**:368–370.

Graczyk TK, Lowenstine LJ, Cranfield MR. 1999. Capillaria hepatica (Nematoda) Infections in Human-Habituated Mountain Gorillas (Gorilla gorilla beringei) of the Parc National de Volcans , Rwanda. Journal of Parasitology **85**:1168–1170. DOI: 10.2307/3285682.

Granjon AC et al. 2020. Estimating abundance and growth rates in a wild mountain gorilla population. Animal Conservation **23**:455-65. DOI: 10.1111/acv.12559.

Gray M, Mcneilage A, Fawcett K, Robbins MM, Ssebide B, Mbula D, Uwingeli P. 2009. Censusing the mountain gorillas in the Virunga Volcanoes : complete sweep method versus monitoring. African Journal of Ecology **48**:588–599.

Gray M, Roy J, Vigilant L, Katie F, Augustin B, Mike C, Prosper U, Innocent M, Edwin K, Robbins MM. 2013. Genetic census reveals increased but uneven growth of a critically endangered mountain gorilla population. Biological Conservation **158**:230–238.

Grueter CC, Li D, Ren B. 2013. Overwintering strategy of Yunnan snub-nosed monkeys : adjustments in activity scheduling and foraging patterns. Primates **54**:125–135.

Guyot K, Follet-Dumoulin A, Lelièvre E, Sarfati C, Rabodonirina M, Nevez G, Cailliez JC, Camus D, Dei-Cas E. 2001. Molecular characterization of Cryptosporidium isolates obtained from humans in France. Journal of Clinical Microbiology **39**:3472–

3480.

Hall TA. 1999. BIOEDIT: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/ NT. Nucleic Acids Symposium Series **41**:95–98.

Han B, Han B, Pan G. 2021. Microsporidiosis in Humans. Clinical Microbiology Reviews **34**:41.

Han B, Weiss LM. 2017. Microsporidia : Obligate Intracellular Pathogens Within the Fungal Kingdom. **5**:5-2. DOI: 10.1128/microbiolspec.FUNK-0018-2016.

Harcourt AH, Parks SA, Woodroffe R. 2001. Human density as an influence on species / area relationships : double jeopardy for small African reserves ? Biodiversity and Conservation **10**:1011–1026.

Hasegawa H. 2018. Spirurids. Pages 164-166 in Modrý D, Pafčo B, Petrželková, KJ, Hasegawa H, editors. Parasites of Apes, An Atlas of Coproscopic Diagnostics. Edition Chimaira Frankfurt Main.

Hasegawa H, Kalousova B, Mclennan MR, Modrý D, Profousova-psenkova I, Shutt-phillips KA, Todd A, Huffman MA, Petrželková KJ. 2016. Strongyloides infections of humans and great apes in Dzanga-Sangha Protected Areas , Central African Republic and in degraded forest fragments in Bulindi , Uganda. Parasitology International **65**:367–370. DOI: 10.1016/j.parint.2016.05.004.

Hastings BE, Kenny D, Lowenstine L., Foster J. 1991. Mountain gorillas and measles: ontogeny of a wildlife vaccination program. Page Proceedings of the American Association of Zoo Veterinarians Annual Meeting. Calgary, Alberta, Canada.

Heyworth MF. 2016. Giardia duodenalis genetic assemblages and hosts. Parasite **13**:5. DOI: 10.1051/parasite/2016013.

Hibbett DS et al. 2007. A higher-level phylogenetic classification of the Fungi. Mycological Research **111**:509–547.

Hickey JR et al. 2019a. Virunga 2015-2016 Surveys: Monitoring mountain gorillas, other select mammals, and illegal acticities. GVTC, IGCP & Partners, Kigali, Rwanda.

Hickey JR et al. 2019b. Bwindi-Sarambwe 2018 Surveys Monitoring Mountain Gorillas,

Other Select Mammals, and Human Activities. Kampala,Uganda. Available from https://igcp.org/content/uploads/2020/09/Bwindi-Sarambwe-2018-Final-Report-2019_12_16.pdf (accessed August 2022).

Hickey JR, Basabose A, Gilardi KV, Greer D, Nampindo S, Robbins MM, Stoinski TS. 2020. Gorilla beringei ssp. beringei, Mountain Gorilla (amended version of 2018 assessment). The IUCN Red List of Threatened Species 2020: e.T39999A176396749. DOI: 10.2305/IUCN.UK.2020-3.RLTS.T39999A176396749.en.

Hogan JN, Miller WA, Cranfield MR, Ramer J, Hassell J, Bosco Noheri J, Conrad PA, Gilardi KVK. 2014. Giardia in mountain gorillas (Gorilla beringei beringei), forest buffalo (Syncerus caffer), and domestic cattle in Volcanoes national park, Rwanda. Journal of Wildlife Diseases **50**:21–30.

Hooshyar H, Rostamkhani P, Rezaeian M. 2015. An Annotated Checklist of the Human and Animal Entamoeba (Amoebida : Endamoebidae) Species- A Review Article. Iran J Parasitol **10**:146–156.

Iseki M. 1979. Cryptosporidium felis sp.n. (Protozoa: Eimeriorina) from the domestic cat. Japanese Journal of Parasitology **28**:285–307.

Iseki M. 1986. Two Species of Cryptosporidium Naturally Infecting House Rats. Japanese Journal of Parasitology **35**:521–526.

Ito A, Eckardt W, Stoinski TS, Gillespie TR, Tokiwa T. 2020. Three new Prototapirella species, Opisthotrichum janus, and Troglocorys cava add to Entodiniomorphida (Ciliophora, Trichostomatia) diversity in mountain gorillas in Rwanda. European Journal of Protistology **76**:125738. DOI: 10.1016/j.ejop.2020.125738.

Jirků-Pomajbíková K, Vlčková K. 2018. Intestinal amoebas. Pages 104-105 in Modrý D, Pafčo B, Petrželková, KJ, Hasegawa H, editors. Parasites of Apes, An Atlas of Coproscopic Diagnostics. Edition Chimaira. Frankfurt am Main.

Johnston AR, Gillespie TR, Rwego IB, Mclachlan TLT, Angela D, Goldberg TL. 2010. Molecular Epidemiology of Cross-Species Giardia duodenalis Transmission in Western Uganda. PLoS Negleted Tropical diseases **4**:9–13. DOI: 10.1371/journal.pntd.0000683.

Jokipii L, Jokipii AM. 1986. Timing of symptoms and oocyst excretion in human cryptosporidiosis. New England Journal of Medicine **315**:1643–1647.

Kalema-Zikusoka G, Rothman JM, Fox MT. 2005. Intestinal parasites and bacteria of mountain gorillas (Gorilla beringei beringei) in Bwindi Impenetrable. Primates **46**:59–63.

Kalema-Zikusoka G, Rubanga S, Mutahunga B, Sadler R. 2018. Prevention of Cryptosporidium and GIARDIA at the Human/ Gorilla/Livestock Interface. Frontiers Public Health **6**:1–7.

Kalpers J, Williamson EA, Robbins MM, McNeilage A, Nzamurambaho A, Lola N, Mugiri G. 2003. Gorillas in the crossfire: Population dynamics of the Virunga mountain gorillas over the past three decades. Oryx **37**:326–337.

Kanyamibwa S. 2013. AIbertine Rift Conservation Status Report. Albertine Rift Conservation Series:2–102.

Karno V. 1990. Protection of endangered gorillas and chimpanzees in international trade: can CITES help. Hastings Int'l & Comp. L. Rev. **14**:989–1015.

Kašičková D, Sak B, Kváč M, Ditrich O. 2009. Sources of potentially infectious human microsporidia: Molecular characterisation of microsporidia isolates from exotic birds in the Czech Republic, prevalence study and importance of birds in epidemiology of the human microsporidial infections. Veterinary Parasitology **165**:125–130.

Katzwinkel-Wladarsch S, Lieb M, Heise W, Loscherl T, Rinder H. 1996. Direct amplification and species determination of microsporidian DNA from stool specimens **I**:373–378.

Kodádková A, Kváč M, Ditrich O, Sak B, Xiao L. 2010. Cryptosporidium muris in a Reticulated Giraffe (Giraffa camelopardalis reticulata). Journal of Parasitology **96**:211–212.

Kumar S, Stecher G, Li M, Knyaz C, Tamura K. 2018. MEGA X: Molecular evolutionary genetics analysis across computing platforms. Molecular Biology and Evolution **35**:1547–1549.

Kváč M et al. 2013. Cryptosporidium scrofarum n . sp . (Apicomplexa : Cryptosporidiidae) in domestic pigs (Sus scrofa). Veterinary Parasitology **191**:218–227.

Kváč M, Hofmannová L, Hlásková L, Květoňová D, Vítovec J, McEvoy J, Sak B. 2014a. Cryptosporidium erinacei n. sp. (Apicomplexa: Cryptosporidiidae) in hedgehogs. Veterinary Parasitology **201**:9–17.

Kváč M, Hofmannová L, Ortega Y, Holubová N, Horčičková M, Kicia M, Hlásková L, Květoňová D, Sak B, McEvoy J. 2017. Stray cats are more frequently infected with zoonotic protists than pet cats. Folia Parasitologica **64**.

Kváč M, Květoňová D, Sak B, Ditrich O. 2009. Cryptosporidium Pig Genotype II in Immunocompetent Crimean-Congo Hemorrhagic Fever, Southwestern Bulgaria. Emerging Infectious Diseases **15**:982–983.

Kváč M, McEvoy J. 2018a. Cryptospordium . Pages 116-117 in Modrý D, Pafčo B, Petrželková, KJ, Hasegawa H, editors. Parasites of Apes, An Atlas of Coproscopic Diagnostics. Edition Chimaira Frankfurt Main.

Kváč M, Mc Evoy. 2018b. Giardia. Pages 98-99 in Modrý D, Pafčo B, Petrželková, KJ, Hasegawa H, editors. Parasites of Apes, An Atlas of Coproscopic Diagnostics. Edition Chimaira Frankfurt Main.

Kváč M, McEvoy J, Stenger B, Clark M. 2014b. Cryptosporidium: parasite and disease. In: S.M. Cacciò and G. Widmer (Eds.), Cryptosporidium. parasite and:237–326.

Kváč M, McEvoy J, Stenger B, Clark M. 2014c. Cryptosporidiosis in other vertebrates. In: S.M. Cacciò and G. Widmer (Eds.), Cryptosporidium. Parasite and Disease. Springer, Wien:237–326.

Lallo MA, Paulista U. 2014. Bacterial and Mycotic Infections in Immunocompromised Hosts : Clinical and Microbiological Aspects.

Leav BA, Mackay M, Ward HD. 2003. Cryptosporidium Species : New Insights and Old Challenges. Food Safety **36**.

Leendertz S, Wich S, Ancrenaz M, Gonder M, Humle T, Leendertz F. 2017. Ebola in great apes – current knowledge, possibilities for vaccination, and implications for

conservation and human health. Mammal Review **47**:98–111.

Li W, Kiulia NM, Mwenda JM, Nyachieo A, Taylor MB, Zhang X, Xiao L. 2011. Cyclospora papionis, Cryptosporidium hominis, and human-pathogenic Enterocytozoon bieneusi in captive baboons in Kenya. Journal of Clinical Microbiology **49**:4326–4329.

Li W, Wan Q, Yu Q, Yang Y, Tao W, Jiang Y, Xiao L. 2016. Genetic variation of mini- and microsatellites and a clonal structure in Enterocytozoon bieneusi population in foxes and raccoon dogs and population differentiation of the parasite between fur animals and humans. Parasitology Research **115**:2899–2904. DOI: 10.1007/s00436-016-5069-3.

Macfie EJ, Williamson EA. 2010. Best Practice Guidelines for Great Ape Tourism. IUCN/SSC Primate Specialist Group (PSG), Gland/Switzeland. Available from http://www.primate-sg.org (accessed August 2022).

Makouloutou P, Philippe P, Nguema M, Fujita S, Takenoshita Y. 2014. Prevalence and genetic diversity of Oesophagostomum stephanostomum in wild lowland gorillas at Moukalaba-Doudou National Park , Gabon Prevalence and genetic diversity of Oesophagostomum stephanostomum in wild lowland gorillas at Moukalaba-Doudou National. Helminthologia **51**:83 – 93.

Mason B et al. 2022. Gastrointestinal symbiont diversity in wild gorilla : A comparison of bacterial and strongylid communities across multiple localities. Molecular Ecology **31**:4127–4145.

Mathis A, Weber R, Deplazes P. 2005. Zoonotic potential of the microsporidia. Clinical Microbiology Reviews **18**:423–445.

Mazet JAK et al. 2020. Human Respiratory Syncytial Virus Detected in Mountain Gorilla Respiratory Outbreaks. EcoHealth **17**:449–460. DOI: 10.1007/s10393-020-01506-8.

Mcgahey DJ, Williams DG, Muruthi P, Loubser DI. 2013. Investigating climate change vulnerability and planning for adaptation : Learning from a study of climate change impacts on the Mountain Gorilla in the Albertine Rift. Nature science **5**:10–17. DOI: 10.4236/ns.2013.55A002.

McNeilage A et al. 2006. Census of the mountain gorilla Gorilla beringei beringei population in Bwindi Impenetrable National Park, Uganda. Oryx **40**:419-427.

Mcneilage AJ. 2001. Diet and habitat use of two mountain gorilla groups in contrasting habitats in the Virungas in Mountain Gorillas: Three Decades of Research at Karisoke.In Robbins, M. M., Sicotte, P. & Stewart, K. J. (Eds.),Mountain Gorilla. Cambridge University Press, Cambridge,UK.:265–292.

Menu E, Davoust B, Mediannikov O, Akiana J, Mulot B, Diatta G, Levasseur A, Raoult D, Bittar F. 2021. Occurrence of Ten Protozoan Enteric Pathogens in Three Non-Human Primate Populations. Pathogens **10**:1–10. DOI: 10.3390/pathogens10030280.

MGVP. 2019, June 17. The Current Ebola Outbreak and Gorilla Conservation. New York Times. Available from https://www.gorilladoctors.org/ebola-and-gorillas/ (accessed August 2022).

Modrý D, Pafčo B, Petrželková KJ, Hasegawa H. 2018. Parasites of Apes, An Atlas of coproscopic Diagnostics. Edition Chimaira. Frankfurt am Main

Mori H, Mahittikorn A, Thammasonthijarern N, Chaisiri K, Rojekittikhun W, Sukthana Y. 2013. Presence of zoonotic Enterocytozoon bieneusi in cats in a temple in central Thailand. Veterinary Parasitology **197**:696–701. DOI: 10.1016/j.vetpar.2013.07.025.

Muhangi D, Gardiner CH, Ojok L, Cranfield MR, Gilardi KVK, Mudakikwa AB, Lowenstine LJ. 2021. Pathological lesions of the digestive tract in free-ranging mountain gorillas (Gorilla beringei beringei). American Journal of Primatology **83**.

Mynářová A, Foitová I, Kváč M, Květoňová D, Rost M, Morrogh-Bernard H, Nurcahyo W, Nguyen C, Supriyadi S, Sak B. 2016. Prevalence of Cryptosporidium spp., Enterocytozoon bieneusi, Encephalitozoon spp. and Giardia intestinalis in wild, semi-wild and captive orangutans (Pongo abelii and Pongo pygmaeus) on Sumatra and Borneo, Indonesia. PLoS ONE **11**:1–15.

Němejc K et al. 2014. Prevalence and diversity of Encephalitozoon spp. and Enterocytozoon bieneusi in wild boars (Sus scrofa) in Central Europe. Parasitology Research **113**:761–767.

Nizeyi J, Cranfield M, Graczyk T. 2002a. Cattle near the Bwindi Impenetrable National Park, Uganda, as a reservoir of Cryptosporidium parvum and Giardia duodenalis for local community and free-ranging gorillas. Parasitology Research **88**:380–385.

Nizeyi JB, Mwebe R, Nanteza A, Cranfield MR, Kalema GRNN, Graczyk TK. 1999. Cryptosporidium sp. and Giardia sp. infections in mountain gorillas (Gorilla gorilla beringei) of the bwindi impenetrable National Park, Uganda. Journal of Parasitology **85**:1084–1088.

Nizeyi JB, Sebunya D, DaSilva AJ, Cranfield MR, Pieniazek NJ, Graczyk TK. 2002b. Cryptosporidiosis in people sharing habitats with free-ranging mountain gorillas (Gorilla gorilla beringei), Uganda. American Journal of Tropical Medicine and Hygiene **66**:442–444.

Nunn CL, Altizer SM. 2005. The Global Mammal Parasite Database : An Online Resource for Infectious Disease Records in Wild Primates. Evolutionary Anthropology **14**:1–2. DOI: 10.1002/EVAN.20041.

Owiunji I, Nkuutu D, Kujirakwinja D, Liengola I, Plumptre A, Nsanzurwimo A, Fawcett K, Gray M, Mcneilage A. 2005. The biodiversity of the Virunga Volcanoes. Available at https://programs.wcs.org/portals/49/media/file/volcanoes_biodiv_survey.pdf (accessed August 2022).

Oyerinde JPO, Ogunbi O, Alonge AA. 1977. Age and Sex Distribution of Infections with Entamoeba histolytica and Giardia intestinal is in the Lagos Population. International Journal of epidemiology **8**:231–234. DOI: 10.12933/therya-21-1156.

Pafčo B. 2018. Parasites of gorillas. in Modrý D, Pafčo B, Petrželková, KJ, Hasegawa H, editors. Parasites of Apes, An Atlas of Coproscopic Diagnostics. Edition Chimaira. Frankfurt am Main.

Pafčo B, Benavides JA, Pšenková-Profousová I, Modrý D, Červená B, Shutt KA, Hasegawa H, Fuh T, Todd AF, Petrželková KJ. 2018. Do habituation, host traits and seasonality have an impact on protist and helminth infections of wild western lowland gorillas? Parasitology Research **116**:3401–3410.

Palacios G et al. 2011. Human Metapneumovirus Infection in Wild Mountain Gorillas,

Rwanda. Emerging Infectious Diseases **17**:711–713. DOI: 10.3201/eid1704/100883.

Palmer CJ, Xiao L, Terashima A, Guerra H, Gotuzzo E, Saldías G, Bonilla JA, Zhou L, Lindquist A, Upton SJ. 2003. Cryptosporidium muris, a rodent pathogen, recovered from a human in Perú. Emerging Infectious Diseases **9**:1174–1176.

Parsons MB, Travis D, Lonsdorf E V, Lipende I, Anthony Roellig DM, Kamenya S, Zhang H, Xiao L, Gillespie TR, Baker S. 2015. Epidemiology and Molecular Characterization of Cryptosporidium spp. in Humans, Wild Primates, and Domesticated Animals in the Greater Gombe Ecosystem, Tanzania. PLoS neglected tropical diseases. DOI: 10.1371/journal.pntd.0003529.

Petrželková KJ et al. 2021. Heterogeneity in patterns of helminth infections across populations of mountain gorillas (Gorilla beringei beringei). Scientific Reports **11**:1–14.

Petrželková KJ et al. 2022. Ecological drivers of helminth infection patterns in the Virunga Massif mountain gorilla population. International Journal for Parasitology: Parasites and Wildlife **17**:174–184.

Petrželková KJ, Smejkalová P, Ćeza V, Pafčo B, Shutt-Phillips K., Jirků-Pomajbíková K, Benavides J, Modry´ D, Čepická I. 2020. Sympatric western lowland gorillas, central chimpanzees and humans are infected with different trichomonads. Cambridge University Press **147**:225–230. DOI: 10.1017/S0031182019001343.

Plumptre AJ, Behangana M, Davenport TRB, Kahindo C, Kityo R, Ndomba E, Nkuutu D, Owiunji I, Ssegawa P, Eilu G. 2003. THE BIODIVERSITY OF THE ALBERTINE RIFT. Albertine Rift technical report No. 3:4–112.

Plumptre AJ, Nangendo G, Ayebare S, Kirunda B, Mugabe H, Nsubuga P, Nampindo S. 2017. Impacts of Climate Change and Industrial Development on the Long-Term Changes in Wildlife Behavior in the Greater Virunga Landscape. Page GVTC-WCS. Kampala,Uganda.

Plumptre AJ, Williamson L. 2001. Conservation-oriented research in the Virunga region:361–390. Cambridge University Press. DOI: 10.1017/CBO9780511661631.015.

Pomajbíková K, Petrželková KJ, Profousová I, Petrášáov J, Modrý D. 2010. Discrepancies in the occurrence of Balantidium coli between wild and captive African great apes. Journal of Parasitology **96**:1139–1144.

Profousová I, Mihaliková K, Laho T, Váradyová Z, Petrželková KJ, Modrý D, Kišidayová S. 2011. The ciliate , Troglodytella abrassarti , contributes to polysaccharide hydrolytic activities in the chimpanzee colon. Folia Microbiol **56**:339–343.

Proudman CJ, Holmes MA, Sheoran AS, Edwards SER, Trees AJ. 1997. Immunoepidemiology of the equine tapeworm Anoplocephala perfoliata : age-intensity profile and age-dependency of antibody subtype responses. Parasitology **114**:89–94.

Pšenková-Profousová I. 2018. Entodiniomorphid ciliates. Pages 107-111. in Modrý D, Pafčo B, Petrželková, KJ, Hasegawa H, editors. Parasites of Apes, An Atlas of Coproscopic Diagnostics. Edition Chimaira. Frankfurt am Main.

R Core Team. 2022. R: A language and environment for statistical computing. R Foundation for Statistical Computing:Vienna, Austria. Available from https://www.r-project.org/.

Reed DH, Frankham R. 2003. Correlation between Fitness and Genetic Diversity. Conservation Biological **17**:230–237.

Reetz J. 1993. [Naturally-acquired microsporidia (Encephalitozoon cuniculi) infections in hens]. Tierarztl Prax **21**:429–435.

Robbins MM et al. 2011. Extreme Conservation Leads to Recovery of the Virunga Mountain Gorillas. PLoS ONE **6**:1–10.

Robbins MM, Gray M, Kagoda E, Robbins AM. 2009. Population dynamics of the Bwindi mountain gorillas. Biological Conservation **142**:2886–2895. DOI: 10.1016/j.biocon.2009.07.010.

Robinson G, Elwin K, Chalmers RM. 2008. Cryptosporidium Genotypes in Human Cases of Diarrhea. Emerging Infectious Diseases **14**:1800–1802.

Rothman J, Bowman DD. 2003. A Review of the Endoparasites of Mountain Gorillas.

Page Companion and Exotic animal Parasitology. International Veterinary Information Service, Ithaca, New York.

Rothman JM, Pell AN, Bowman DD. 2008. Host-parasite ecology of the helminths in mountain gorillas. Journal of Parasitology **94**:834–840.

Roy J, Vigilant L, Gray M, Wright E, Kato R, Kabano P, Basabose A, Tibenda E, Kühl HS, Robbins MM. 2014. Challenges in the use of genetic mark-recapture to estimate the population size of Bwindi mountain gorillas (Gorilla beringei beringei). BIOLOGICAL CONSERVATION **180**:249–261. DOI: 10.1016/j.biocon.2014.10.011.

Ryan U, Fayer R, Xiao L. 2014. Cryptosporidium species in humans and animals: Current understanding and research needs. Parasitology **141**:1667–1685.

Sak B et al. 2013. Long-Term Monitoring of *Microsporidia, Cryptosporidium* and *Giardia* Infections in Western Lowland Gorillas (Gorilla gorilla gorilla) at Different Stages of Habituation in Dzanga Sangha Protected Areas, Central African Republic. PLoS ONE **8**:10.

Sak B, Brady D, Pelikánová M, Květoňová D, Rost M, Kostka M, Tolarová V, Hůzová Z, Kváč M. 2011a. Unapparent microsporidial infection among immunocompetent humans in the Czech Republic. Journal of Clinical Microbiology **49**:1064–1070.

Sak B, Kváč M. 2018. Microspordia. Pages 118-119 in Modrý D, Pafčo B, Petrželková, KJ, Hasegawa H, editors. Parasites of Apes, An Atlas of Coproscopic Diagnostics. Edition. Chimaira. Frankfurt am Main

Sak B, Kváč M, Hanzlíková D, Cama V. 2008. First report of *Enterocytozoon bieneusi* infection on a pig farm in the Czech Republic. Veterinary Parasitology **153**:220–224.

Sak B, Kváč M, Kučerová Z, Květoňová D, Saková K. 2011b. Latent microsporidial infection in immunocompetent individuals - a longitudinal study. PLoS Neglected Tropical Diseases **5**:1–5.

Sak B, Kváč M, Květoňová D, Albrecht T, Piálek J. 2011c. The first report on natural Enterocytozoon bieneusi and Encephalitozoon spp. infections in wild East-European

House Mice (Mus musculus musculus) and West-European House Mice (M. m. domesticus) in a hybrid zone across the Czech Republic-Germany border. Veterinary Parasitology **178**:246–250.

Sak B, Kváč M, Petrželková K, Květoňová D, Pomajbíková K, Mulama M, Kiyang J, Modrý D. 2011d. Diversity of microsporidia (Fungi: Microsporidia) among captive great apes in European zoos and African sanctuaries: Evidence for zoonotic transmission? Folia Parasitologica **58**:81–86.

Sak B, Petrželková KJ, Květoňová D, Mynářová A, Pomajbíková K, Modrý D, Cranfield MR, Mudakikwa A, Kváč M. 2014. Diversity of Microsporidia , *Cryptosporidium* and *Giardia* in Mountain Gorillas (Gorilla beringei beringei) in Volcanoes National Park , Rwanda. PLoS ONE **9**:1–7.

Saková K, Sak B, Ditrich O, Kváč M. 2006. Humoral response of chicken infected with the microsporidium Encephalitozoon hellem. Parasitology Research **98**:488–492.

Salyer SJ, Gillespie TR, Rwego IB, Chapman CA, Goldberg TL. 2012a. Epidemiology and Molecular Relationships of Cryptosporidium spp . in People , Primates , and Livestock from Western Uganda. PLoS Negl Trop Dis **6**:2–7.

Salyer SJ, Gillespie TR, Rwego IB, Chapman CA, Goldberg TL. 2012b. Epidemiology and Molecular Relationships of Cryptosporidium spp . in People , Primates , and Livestock from Western Uganda **6**:2–7.

Sandbrook C, Semple S. 2006. The rules and the reality of mountain gorilla Gorilla beringei beringei tracking : how close do tourists get ? Oryx **40**:428–433.

Santín M, Cortés JA, Fayer R. 2013. A large scale molecular study of Giardia duodenalis in horses from Colombia. Veterinary Parasitology **196**:31–36. Elsevier B.V. ADOI: 10.1016/j.vetpar.2013.02.006.

Santín M, Fayer R. 2011a. Research in Veterinary Science Microsporidiosis : Enterocytozoon bieneusi in domesticated and wild animals **90**:363–371.

Santín M, Fayer R. 2011b. Microsporidiosis: Enterocytozoon bieneusi in domesticated and wild animals. Research in Veterinary Science **90**:363–371. W.B. Saunders.

Sawyer I. 2018. Kidnappings, Killings in DR Congo's Virunga National Park. Available

from https://www.hrw.org/news/2018/05/15/kidnappings-killings-dr-congos-virunga-national-park.

Schaller GB. 1963. The Mountain Gorilla: Ecology and Behavior.

Schmidt GD, Roberts LS, Janovy john R. 2009. Foundations of Parasitology. McGraw-Hill, New York.

Schnell K, Collier S, Derado G, Yoder J, Gargano JW. 2016. Giardiasis in the United States – an epidemiologic and geospatial analysis of county-level drinking water and sanitation data , 1993 – 2010. Journal of Water and Health:267–279.

Shabahat E. 2018. Rwandan people and mountain gorillas face changing climate together. Page Rwandan people and mountain gorillas face changing climate together. Available from https://news.mongabay.com/2018/06/rwandan-people-and-mountain-gorillas-face-changing-climate-together/ (accessed August 2022).

Shoemaker T et al. 2011. Reemerging Sudan Ebola Virus Disease in. Emerging Infectious Diseases **18**:1480–1483.

Shutt K et al. 2014. Effects of habituation , research and ecotourism on faecal glucocorticoid metabolites in wild western lowland gorillas : Implications for conservation management. Biological Conservation **172**:72–79. DOI: 10.1016/j.biocon.2014.02.014.

Sinayitutse E, Modry D, Slapeta J, Nyiramana A, Mudakikwa A, Muvunyi R, Eckardt W. 2021. Daily defecation outputs of mountain gorillas (Gorilla beringei beringei) in the Volcanoes National Park, Rwanda. Primates **62**:311–320.

Sleeman JM, Meader LL, Mudakikwa AB, Foster JW, Patton S. 2000. Gastrointestinal Parasites of Mountain Gorillas (Gorilla gorilla beringei) in the Parc National des Volcans, Rwanda. Journal of Zoo and Wildlife Medicine **31**:322–328.

Spelman A, Lucy H, Kirsten VK. 2013. Respiratory Disease In Mountain Gorillas (Gorilla Beringei Beringei) In Rwanda , 1990 – 2010 : Outbreaks , Clinical Course , And Medical Management. Journal of Zoo and Wildlife Medicine **44**:1027–1035. DOI: 10.1638/2013-0014R.1.

Sulaiman IM, Fayer R, Bern C, Gilman RH, Trout JM, Schantz PM, Das P, Lal AA, Xiao

L. 2003a. Triosephosphate Isomerase Gene Characterization and Potential Zoonotic Transmission of Giardia duodenalis **9**:15–17.

Sulaiman IM, Fayer R, Lal AA, Trout JM, Schaefer FW, Xiao L. 2003b. Molecular characterization of microsporidia indicates that wild mammals harbor host-adapted Enterocytozoon spp. as well as human-pathogenic Enterocytozoon bieneusi. Applied and Environmental Microbiology **69**:4495–4501.

Thellier M, Breton J. 2008. Enterocytozoon Bieneusi in Human and Animal, Focus on Laboratory Identification and Molecular Epidemiology. Parasite **15**:349–358.

Thompson RCA, Ash A. 2016. Molecular epidemiology of Giardia and Cryptosporidium infections. Infection, Genetics, and Evolution **40**:315–323.

Thompson RCA, Palmer CS, O'Handley R. 2008. The public health and clinical significance of Giardia and Cryptosporidium in domestic animals. Veterinary Journal **177**:18–25.

Thorne JH, Seo C, Basabose A, Gray M, Belfiore NM, Hijmans RJ. 2013. Alternative biological assumptions strongly influence models of climate change effects on mountain gorillas. Ecosphere **4**:1–17.

Towner JS et al. 2008. Newly Discovered Ebola Virus Associated with Hemorrhagic Fever Outbreak in Uganda. PLoS Pathogens **4**:3–8.

UNEP. 2003. Report on the status of the Mountain Gorilla (Gorilla gorilla beringei). UNEP World Conservation Monitoring Centre.

Valigurová A, Hofmannová L, Koudela B, Vávra J. 2007. An Ultrastructural Comparison of the Attachment Sites Between Gregarina steini and Cryptosporidium muris ´ ,. Journal of Eukaryotic Microbiology **54**:495–510.

van del Hoek Y, Emmanuel F, Eckardt W, Kwizera I, Derhé M, Caillaud D, Stoinski tara S, Tuyisingize D. 2019. Recent decline in vegetative regeneration of bamboo (Yushania alpina), a key food plant for primates in Volcanoes National Park, Rwanda. Scientific Reports **9**:1–10.

Verweij JJ, Blange RA, Templeton K, Schinkel J, Brienen EAT, Rooyen MAA Van, Lieshout L Van, Polderman AM. 2004. Cryptosporidium parvum in fecal samples

by using Multiplex real-time PCR. Journal of clinical microbiology **42**:1220–1223.

Vetterling JM, Jervis HR, Thomas MC, Sprinz H. 1971. Cryptosporidium wrairi sp. n. from the guinea pig Cavia porcellus, with an emendation of the genus. journal of protozoology **18**:243–247.

Vlčková K, Kreisinger J, Pafčo B, Čížková D, Tagg N, Hehl AB, Modrý D. 2018. Diversity of Entamoeba spp. in African great apes and humans: an insight from Illumina MiSeq high-throughput sequencing. International Journal for Parasitology **48**:519–530.

Watts DP. 1984. Composition and Variability of Mountain Gorilla Diets in the Central Virungas. American Journal of Primatology **7**:323–356.

Watts DP. 1998. Long-Term Habitat Use by Mountain Gorillas (Gorilla gorilla beringei). 1 . Consistency, Variation, and Home Range Size and Stability. International Journal of Primatology **19**:651–680.

Weber AW, Vedder A. 1983. Population Dynamics of the Virunga Gorillas : **26**:341–366.

Wells K, Clark NJ. 2019. Host Speci fi city in Variable Environments. Trends in Parasitology **35**:452–465.

Williamson EA, Fawcett KA. 2008. Long-term research and conservation of the Virunga mountain gorillas. Science and Conservation in African Forests: The Benefits of Long-term Research:213–229. Cambridge University Press, Cambridge,UK.

Williamson EA, Feistner AC. 2011. Habituating primates : processes , techniques , variables and ethics. Field and Laboratory Methods in Primatology: A Practical Guide:33–50. Cambridge University Press.

Winters AD, Faisal M. 2014. Molecular and ultrastructural characterization of Dictyocoela diporeiae n . sp . (Microsporidia), a parasite of Diporeia spp . (Amphipoda , Gammaridea). Parasite **21**.

Wolk DM, Schneider SK, Wengenack NL, Sloan LM, Rosenblatt JE. 2002. Real-Time PCR Method for Detection of Encephalitozoon intestinalis from Stool Specimens **40**:3922–3928.

Xiao L, Feng Y. 2017. Molecular epidemiologic tools for waterborne pathogens

Cryptosporidium spp . and Giardia duodenalis. Food and Waterborne Parasitology **8**–**9**:14–32. DOI: 10.1016/j.fawpar.2017.09.002.

Xiao L, Morgan UNAM, Limor J, Escalante A, Arrowood M, Shulaw W, Thompson RCA, Fayer R, Lal AA, Carolina N. 1999. Genetic Diversity within Cryptosporidium parvum and Related Cryptosporidium Species Michigan. American Society for Microbiology **65**:3386–3391.

Xu C, Ma X, Zhang H, Zhang XX, Zhao JP, Ba HX, Rui-Du, Xing XM, Wang QK, Zhao Q. 2016. Prevalence, risk factors and molecular characterization of Enterocytozoon bieneusi in raccoon dogs (Nyctereutes procyonoides) in five provinces of Northern China. Acta Tropica **161**:68–72. DOI: 10.1016/j.actatropica.2016.05.015.

Xue Y et al. 2016. Mountain gorilla genomes reveal the impact of long-term population decline and inbreeding. Science **348**:242–246.

Zahedi A, Paparini A, Jian F, Robertson I, Ryan U. 2016. Public health significance of zoonotic Cryptosporidium species in wildlife: Critical insights into better drinking water management. International Journal for Parasitology: Parasites and Wildlife **5**:88–109. DOI: 10.1016/j.ijppaw.2015.12.001.

Zhao W, Zhang W, Yang Z, Liu A, Zhang L, Yang F, Wang R, Ling H. 2015. Genotyping of Enterocytozoon Bieneusi in Farmed Blue foxes (Alopex lagopus) and Raccoon dogs (Nyctereutes procyonoides) in China. PLoS ONE **10**:1–9.