INTERACTIONS BETWEEN PRIMATES AND PARASITES IN A WILD COMMUNITY

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THESIS ABSTRACT

Neglected tropical diseases (NTDs) are a group of tropical parasites that affect ~1.5 billion people worldwide. Despite their ubiquity, they often do not kill those they infect and so are frequently overlooked in research and policy. These parasites should concern public health and wildlife conservation researchers, however, because their burden is predicted to greaten with changes in climate and land use. Our world is also becoming increasingly connected, which results in higher risk of disease transmission between wildlife, livestock, and people. In this thesis, I explore how parasites are transmitted among a wild primate community and adjacent rural settlements using two groups of NTDs: soil transmitted helminths and vector-borne blood parasites. Host-parasite theory predicts that the breadth of hosts that a parasite can infect relates to parasite life history. Parasites with slow generation times and complex physiology (like soil transmitted helminths) are likely to have narrow host ranges. In contrast, parasites with rapid generation times and high potential to encounter new hosts (like vector-transmitted blood parasites) are likely to have broad host ranges. My research took place in Kibale National Park, a tropical rainforest in Uganda. Kibale provides a unique opportunity to test theoretical predictions on host specificity and transmission because the diurnal primate community of Kibale is composed of eight species that differ in life history and phylogenetic relatedness. In addition, Kibale is surrounded by an extremely dense congregation of human settlements, which may facilitate direct transmission between wild primates and human populations. Chapters 2, 3 and 4 each examine a parasite that commonly infects both wild primates and humans. Using molecular methods, I show that two soil transmitted helminths (*Oesophagostomum* and *Trichuris*) have broad host ranges, whereas two vector-borne blood parasites (*Plasmodium* and *Hepatocystis*) have narrow host ranges. These results run counter to theoretical expectation based on life history, which demonstrates the complexity of host-parasite dynamics at the system level. Chapter 4 addresses the consequences NTDs in wildlife. I tested how infection correlates with host behaviour using a broadly transmitted parasite (*Trichuris*) and the endangered red colobus monkey (*Procolobus rufomitratus*). I combined red colobus activity data with parasite infection data, and show that infected animals exhibit signs of illness. Overall, my work highlights the significance of NTDs – not only are some freely transmitted between humans and wildlife, but they appear to pose a health risk to both.

RÉSUMÉ DE THÈSE

Les maladies tropicales négligées (MTNs) sont un groupe de parasites tropicaux qui affectent environ 1.5 milliards d'individus dans le monde. Malgré leur grande prévalence, les MTNs ne causent habituellement pas la mort, ainsi leur tendance à être ignorées en recherche et en gestion de la santé. Les chercheurs en santé publique et conservation de la faune devraient cependant porter davantage attention à ces parasites car on prédit que l'impact de ceux-ci sera amplifié par les changements climatiques et d'utilisation du sol. Notre monde est aussi de plus en plus connecté, induisant ainsi des risques accrus de transmission de maladies entre la faune, le bétail, et les humains. Dans cette thèse, je m'intéresse à la transmission de parasites entre une communauté de primates sylvestres et les villages ruraux avoisinants en me concentrant sur deux groupes de MTNs : les vers parasites transmis par le sol et les parasites sanguins à transmission vectorielle. La théorie des interactions hôte-pathogène prédit que la quantité d'hôtes qu'un parasite puisse infecter est reliée aux traits d'histoire de vie du parasite. Par exemple, on prédit que les parasites avec un temps de génération élevé et une physiologie complexe (tel que les vers parasites transmis par le sol) devraient avoir peu d'hôtes potentiels, alors que les parasites avec un temps de génération moindre et une grande capacité à être mis en contact avec de nouveaux hôtes (tel que les parasites sanguins à transmission vectorielle) devraient avoir une grande quantité d'hôtes potentiels. Mes travaux de recherche ont été réalisés dans le Parc National de Kibale, une forêt tropicale en Ouganda. Kibale est un endroit idéal pour tester les prédictions théoriques à propos de la transmission des maladies et de la spécificité des hôtes car sa communauté de primates diurnes est composée de huit espèces qui diffèrent en termes d'histoire de vie et de relation phylogénétique. En plus, Kibale est entouré d'une multitude de villages très rapprochés, ce qui pourrait faciliter la transmission directe de maladies entre les primates et les populations humaines. Les chapitres 2, 3 et 4 examinent chacun un parasite qui infecte communément autant les primates que les humains. En utilisant des techniques de biologie moléculaire, je montre que deux vers parasites transmis par le sol (*Oesophagostomum* et *Trichuris*) ont un nombre limité d'hôtes, alors que deux parasites sanguins à transmission vectorielle (*Plasmodium* et *Hepatocystis*) ont une grande quantité d'hôtes. Ces résultats sont à l'opposé des prédictions théoriques découlant de la théorie de l'histoire de vie, ce qui démontre la complexité des dynamiques hôtes-parasite au niveau du système. Le chapitre 4 s'intéresse aux conséquences des MTNs sur la faune. J'examine les effets d'un parasite commun (*Trichuris*) sur

le comportement d'une espèce de primate menacée, la colobe rouge (*Procolobus rufomitratus*). Je combine des observations comportementales d'une population de colobes rouges avec des données d'infection au parasite *Trichuris* pour montrer que les animaux infectés démontrent des symptômes de maladie. En somme, mon travail souligne l'importance des MTNs; non seulement sont-elles facilement transmises entre les humains et la faune, mais en plus elles semblent poser un risque pour la santé de chacun.

DEDICATION

This work is dedicated to my parents, Anoop and Laila Ghai, who lived in Kenya from birth. They left everything they loved behind to move to Canada, in the hopes that doing so would afford my brother and I better opportunities in life.

I hope this document gives them reassurance that all they sacrificed for us was not in vain.

Mom & Dad - Thank you for passing on your love of Africa to me. You were right – it gets in your blood. This is one affliction I hope to never be cured of.

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PREFACE

Thesis Format and Style

This thesis is written in a manuscript-based format. It consists of a set of five manuscripts. Two of these manuscripts comprise one chapter (Ch. 4), and I am the lead author on one of these two manuscripts. I am the lead author on Chapters 2, 3, and 5. The style of each chapter follows the scientific journal *Ecology Letters*. All chapters use data collected from my own fieldwork and long-term study in Kibale National Park, Uganda.

Chapter 2: Ghai R.R., Chapman C.A., Omeja P.A., Davies T.J., Goldberg T.L. (2014). Nodule worm infection in humans and wild primates in Uganda: cryptic species in a newly identified region of human transmission. *PLoS Neglected Tropical Diseases*, 8, e2641.

Chapter 3: Ghai R.R., Simons N.D., Chapman C.A., Omeja P.A., Davies T.J., Ting N., and Goldberg T.L. (2014). Hidden population structure and cross-species transmission of whipworms (*Trichuris* sp.) in humans and non-human primates in Uganda. *PLoS Neglected Tropical Diseases*, 8, e3256.

Chapter 4 Part I: Thurber M.I., Ghai R.R., Hyeroba D., Weny G., Tumukunde A., Chapman C.A., Wiseman R.W., Dinis J., Steeil J., Greiner E.C., Freidrich T.C., O'Connor D.H., Goldberg T.L. (2013). Co-infection and cross-species transmission of divergent *Hepatocystis* lineages in a wild African primate community. *International Journal for Parasitology*, 43, 613-619.

Part II: Ghai R.R., Thurber M.I., El Bakry A., Chapman C.A., Goldberg T.L. (In preparation for *Malaria Journal*) Multi-method assessment of patients with febrile illness reveals over-diagnosis of malaria in rural Uganda.

Chapter 5: Ghai R.R., Fugere V.F., Chapman C.A., Goldberg T.L., and Davies T.J. (Under Review,*Proceedings of the Royal Society B: Biological Sciences*). Sickness behaviour associated with
non-lethal parasite infections in wild primates.

Contribution of Co-authors

This thesis is comprised of my original contributions. During my PhD, I received guidance and assistance from a number of collaborators that provided insight, technical expertise, and equipment/materials. All data generated from fieldwork in and around Kibale National Park was conducted with the assistance of skilled personnel, which included field assistants, veterinarians, doctors and nurses. However, I was responsible for generating the research question, producing a scientific framework, analysing the data, and writing the manuscript for all four thesis chapters.

Chapter 2: This work was conducted with the skilled personnel of the Kibale Monkey Project (KMP), which is directed by C.A. Chapman. The KMP manager, P.A. Omeja, provided support in the form of resources and technical assistance at the field site. All laboratory work was conducted under the supervision of T.L. Goldberg. Assistance and analytical advice was provided by T.J. Davies. I collected all of the samples for this project with the assistance of local personnel.

Chapter 3: This work was conducted in collaboration with the same individuals, in the same roles as above (Chapter 2). In addition, N.D. Simons and his supervisor, N. Ting, provided valuable expertise and analytical advice specifically pertaining to the molecular biology of this parasite. I collected all of the samples for this project with the assistance of local personnel.

Chapter 4 Part I: This work was conducted as part of the Kibale Ecohealth Project (KEP), which is directed by T.L. Goldberg and C.A. Chapman. Skilled personnel (G.A. Weny, D. Hyeroba, A. Tumukunde) were required for the invasive sampling procedure, as this project required darting wild primates and collecting blood samples from anaesthetized animals. M.I. Thurber was recruited to assist me in screening blood samples for intra-erythrocytic infections using PCR-based methods (adapted from J. Steeil's unpublished work). However, she was granted primary authorship for this research because she subsequently also used deep sequencing approaches to further explore the data and more fully answer our research questions. Deep sequencing data was generated in collaboration with experts R.W. Wiseman, J. Dinis, T.C. Freidrich, and D.H. O'Connor. Finally, my blood slide diagnoses were confirmed by E.C. Greiner.

Chapter 4 Part II: This work was conducted as part of the KEP, which is directed by T.L. Goldberg and C.A. Chapman. M.I. Thurber was involved in the design of the novel PCR method used in this study. A. El Bakry provided valuable guidance in public health procedures and sampling protocols, and also confirmed my blood slide diagnoses. I collected all of the samples for this project with the assistance of medical professionals.

Chapter 5: This work was conducted using long-term data from a habituated primate group, which was generously provided by C.A. Chapman. The project also used the skilled personnel of the KMP, which is directed by C.A. Chapman. V. Fugere contributed valuable knowledge about statistical modelling. T.J. Davies and T.L. Goldberg provided analytical advice and T.J. Davies assisted with manuscript development. I collected focal sample data, and was involved in both long-term activity data collection and helminth parasite data collection and examination.

Acknowledgements

Studying animals in the wild has been a lifelong dream of mine. I am incredibly grateful for the many people who have helped me get to this stage. Firstly, I would like to thank Colin Chapman for taking a chance on me and inviting me to conduct my research at McGill. Colin's incredible knowledge, connections, and long-term research site in Kibale National Park was the bread and butter of this thesis. The Chapman lab, in particular, Aerin Jacob, Kaia Tombak, Jan Gogarten, Johanna Bleecker, Michael Wasserman, Rafael Reyna-Hurtado, Julie Teichroeb and Chesley Walsh have all been exceptional colleagues and even better friends. Very special thanks to Aerin, my academic support system, for being there from the very start to the very end.

I would also like to thank my supervisor Jonathan Davies for his ongoing support and encouragement. I owe the completion of this thesis to Jonathan, who never wavered in his belief of my abilities or the merits of this research. Being a member of the Davies lab has been a privilege and an intellectual high point. Thank you to Tammy Elliott, Maxwell Farrell, Chelsea Chisholm, Hercules Araclides, and Stephanie Shooner for your contribution to this work, and for your friendship. You're the best lab-mates that anyone could hope for.

I could not have accomplished this work without Tony Goldberg at the University of Wisconsin-Madison, who has patiently guided me through the world of primate infectious diseases from the very start. Tony, I cannot thank you enough for taking me under your wing, for tirelessly believing in me, and for instilling in me a wonder for pathogens and discovery that will never fade. To Tony's lab, I would like to give special thanks to Aleia McCord, Sarah Paige, Tavis Anderson and Mary Thurber for their effortlessly brilliant advice and unfailing generosity.

I am grateful for the advice and expertise of many individuals who helped me develop as a student and a scholar. This includes Drs. Lauren Chapman, Marilyn Scott, Dwight Bowman, and Jessica Rothman. I would also like to thank Patrick Omeja and Dennis Twinomugisha, the Directors of the Kibale Fish and Monkey Project, for their immense assistance in every aspect of my projects' field components. Patrick, in particular, deserves special thanks for not letting my clumsiness and poor immunity have the better of me in Kibale.

This research was possible because of the incredibly skilled assistants that are part of the Kibale Monkey Project, without whom I would still be wandering, lost, in a tropical rainforest. A heartfelt thank you goes to my friends and closest assistants, Robert Araali Basaija, Richard Apuuli Kasarengi, and Peter Atwooki Tuhairwe.

For permission to conduct this work, I thank the Office of the President, the Uganda National Council for Science and Technology, and the Uganda Wildlife Authority. Also, to the Makerere University Biological Field Station for the accommodation, administrative, and logistical support they provide, and for allowing generations of students to come and be inspired by the tremendous ecosystem in Kibale.

The Biology Department of McGill, which has been not only the home base of my academic pursuits but the funding source for many of my endeavours, also deserves special recognition. For five years, I was fortunate to be part of a dynamic and intimidatingly accomplished group of scholars. I have grown tremendously as both a scientist and person because of this department. I would like to thank the Biology Graduate Students Association for providing me with ample opportunity to get to know my peers and faculty members, and the many that do so much to enrich the lives and careers of graduate students. I'd especially like to thank Susan Bocti, Ancil Gittens, and Marisa Masterbardino for their behind-the-scenes work that has made the progression through my PhD effortless. I would also like to thank the agencies that invested in me during this work, including: the Natural Sciences and Engineering Research Council of Canada, McGill University, the McGill Biology Department, the Quebec Centre for Biodiversity Science, the Fond québécois de la recherche sure la nature et technologies, and the National Geographic Society.

Finally, I would like to thank my friends and family for endless laughter, love and support. I could not have done this without you. To Keith Simms, my amazing partner and best friend, I owe my sanity. Thank you for your love that's kept me going, for making me laugh and lifting my spirits, and for always believing in me.

CHAPTER 1

Introduction and Literature Review

1.1 Overview

Parasites are capable of strongly influencing wildlife populations. Most obviously, infectious disease outbreaks increase host mortality which can potentially devastate host populations. In the last decade, a number of serious outbreaks have occurred in wildlife, including Ebola virus in great apes (Huijbergts & Wachter 2003; Bermejo *et al.* 2006), phocine distemper virus in seals (Jensen *et al.* 2002), and chytrid fungus in amphibians (Dobson & Foufopoulos 2001; Wake & Vredenburg 2008). Such epidemics have high mortality, and provide clear examples of the effects of parasites on host populations. However, endemic parasites can also regulate populations by reducing host reproductive success or fitness (Minchella & Scott 1991). A classic study of red grouse (*Lagopus lagopus*) and parasitic nematodes showed that heavily infected individuals were less fecund and more susceptible to predation – a relationship that is responsible for grouse population cycling (Dobson & Hudson 1992; Hudson *et al.* 1992a; Hudson *et al.* 1992b). Experimentally removing nematodes reduced systemic population crashes, suggesting that parasites were the regulatory force in this system (Hudson *et al.* 1998). With several wildlife studies showing the impact of parasites on host survival and fecundity, parasites are now recognized as intrinsic regulatory factors that are as important as predation or competition in some systems (Anderson 1978; Anderson & May 1979; Scott 1988; Hudson *et al.* 2002).

1.2 Parasite Transmission in Populations

Since initially identifying the significance of parasites in natural populations, a robust literature has developed that explores the factors influencing parasite transmission. By convention, these are often separated into host, parasite, and abiotic factors.

1.2.1 Host factors

Contrary to the assumptions of founding epidemiological models, individual hosts vary in both their exposure and susceptibility to parasites (Anderson & May 1992). These variations result from myriad factors, including host traits and behaviour. The relationships between host demography (*e.g.*, age, sex, body size, condition, and genetics) and parasite transmission have been examined in a number of wildlife studies (see reviews by Hudson *et al.* (2002) and Nunn and Altizer (2006)). In general, larger bodied, stressed, or poor-condition hosts are most likely to be burdened with parasites (Hudson *et al.* 2002). Host behaviours can also increase or decrease

parasite transmission. Some hosts can reduce parasitism through behavioural change. In mammals, "anti-parasite" behaviours include grooming, tail flipping, grouping, avoiding contaminated areas, and selective foraging (Hart 1990, 1992; Mooring & Hart 1992; Hart 1994; Mooring *et al.* 2004). Failure to behaviourally defend against parasites can increase both the likelihood and intensity of infection (Daly & Johnson 2011).

Recently, differences in host behaviour have been explored through social networks, which mathematically quantify contact among individuals or groups within a population. Studies using social networks generally conclude that individuals with higher frequencies of conspecific contacts are more likely to both acquire and transmit infections (Meyers 2007; MacIntosh *et al.* 2012; Rushmore *et al.* 2013). Lastly, host factors acting at the group level, such as host population density, can also impact transmission. A number of studies have found that higher host density and larger group sizes can sustain more parasites, and are therefore positively correlated with greater parasite diversity and abundance (Côté & Poulin 1995; Arneberg 2002; Kamiya *et al.* 2014).

1.2.2 Parasite factors

While less understood than host traits, heterogeneity in parasite traits also plays a role in transmission. Parasitic lifestyles have evolved independently across all major kingdoms, and so parasites show an extraordinary diversity in lifecycle and modes of transmission. Some studies have shown that the taxonomic grouping of a parasite can influence both the mode and breadth of transmission (Pedersen *et al.* 2005). An interesting avenue of research has investigated how even within a parasite species, heterogeneity between individuals also alters transmission. Empirical evidence of this has come from studies of mice infected with the malaria parasite *Plasmodium chaubaudi*. Infections in mice administered genetically mixed-clone infections were more severe but also persisted longer than infections in mice given genetically identical parasite clones. Mixed-clone infections were thought to allow prolonged transmission because each genetic variant required recognition by the immune system – a process that takes longer than it would in a monotypic infection (Taylor *et al.* 1998). Conversely, mixed genotype infections might compete with each other inside the host, such that parasites with slower transmission are outcompeted by more exploitative parasites. In this case, parasite virulence is expected to

increase through selection for the rapidly transmitting parasite, resulting in faster overall transmission (at least in the short term) (Read & Taylor 2001). These results are supported by empirical studies on mice (de Roode *et al.* 2005), and suggest that the composition of parasite variants within a single host can greatly alter larger-scale transmission dynamics.

1.2.3 Abiotic factors

Factors that do not directly pertain to host or parasite may also influence transmission. For example, environmental variables like temperature and rainfall have well-studied effects on the persistence and longevity of parasites; especially those with life stages that involve direct development in the environment or vector transmission (Nunn & Altizer 2006). At the regional scale, seasonal variation in climate can cause fluctuating disease patterns within systems (Nelson *et al.* 2002). At the global scale, a warming world is predicted to increase both the abundance and distribution of parasites (Harvell *et al.* 2002).

With research showing strong associations between environmental factors and hosts, vectors and parasites, the field of landscape epidemiology has emerged to identify how geographic variation affects patterns of infection. The premise of landscape epidemiology is that environments are heterogeneous, so including spatial information can help understand or even predict dynamics of transmission (Ostfeld *et al.* 2005). For example, an irregular pattern of rabies spread in racoons across Connecticut was explained by rivers that acted as semi-permeable barriers to transmission (Smith *et al.* 2002). Recent research in the field of landscape genetics has also shown that the environment can shape the genetics of hosts and parasites, and can therefore be used to better understand patterns of disease transmission (Biek & Real 2010).

1.2.4 Conclusions

Despite the burgeoning field of host-parasites interactions, much traditional ecological research has focused on transmission between one host species and one parasite species. However, wildlife species typically host many parasites, and parasites are often capable of infecting more than one species. While the ‘one host, one parasite’ focus has laid the groundwork for studying host-parasite interactions, natural systems are much more complex. My research builds upon

these concepts to investigate the underlying drivers of parasite transmission in a community of wild primates.

1.3 Parasite Transmission in Communities

In recent years, research has considered parasites in community-level processes. Incorporating parasites into food web structure, for example, reveals new linkages that affect the way we interpret the diversity and complexity of trophic systems (Dunne *et al.* 2013). Indeed, a predominant focus of research seeks to understand the effects of parasites in host communities.

1.3.1 Consequences of Single-Host Parasites

Parasites that infect only one host (*i.e.*, single-host or "specialist" parasites) can have strong impacts on communities. A study of benthic marine invertebrates (cockles, *Austrovenus stutchburyi*) showed that trematode infection reduced the cockle's ability to bury into sediment. The authors note that since cockles are "ecosystem engineers" (*i.e.*, animals capable of modulating their habitat structure), their infection can substantially change the dynamics of communities (Mouritsen & Poulin 2005). Experimental studies showed that the resulting changes in sediment disturbance and substrate availability changed both the structure and function of the soft-bottomed animal community (Mouritsen & Poulin 2005). While this research demonstrates that single-host parasites can result in community-level change through cascading effects, other field examples are rare. It may be that there is difficulty in acquiring this information, or that community-level effects from single-host parasites are the exception rather than the norm. Conversely, there is a considerable body of evidence suggesting that multi-host parasites are capable of causing large-scale changes to communities.

1.3.2 Consequences of Multi-Host Parasites

Parasites that can infect multiple host species (*i.e.*, multi-host or "generalist" parasites) are a concern to both wildlife and human health for three important reasons. First, generalist parasites pose an elevated risk of emerging in new hosts because of their propensity to adapt to multiple species. Indeed, studies that have examined risk factors associated with human disease emergence have shown that nearly two-thirds of emerging parasites can infect multiple hosts (Cleaveland *et al.* 2001; Taylor *et al.* 2001). Secondly, generalist parasites are the probable

culprits of parasite-induced extinctions because cross-species transmission allows the parasite to be maintained within systems, even as populations of one species decline (Pedersen *et al.* 2007). For example, domestic dogs are a common host of rabies, and were implicated in "spillover" mortalities of at-risk African wild dogs in Tanzania (Gascoyne *et al.* 1993). Finally, multi-host parasites may be less constrained to evolve reduced virulence. Single-host parasites must balance virulence with transmission because excessive virulence will kill the host before the parasite achieves maximal transmission (Lipsitch & Moxon 1997; Woolhouse *et al.* 2001). This selective constraint may be less stringent when multiple hosts can be infected because the pool of available hosts is larger, and because there could be little consequence of virulence in "dead end" hosts (Woolhouse *et al.* 2001). Empirical studies have both agreed with and opposed this prediction, however (Leggett *et al.* 2013). Contrasting results may be a consequence of system-specific dynamics or oversimplifications in the costs of generalism (Garamszegi 2006; Leggett *et al.* 2013). Nevertheless, these three reasons suggest that multi-host parasites create networks of interaction between their hosts that can strongly influence community dynamics, even if they are difficult to predict.

1.3.3 Predicting Host Specificity

Given that multi-host parasites pose an increased risk to both humans and wildlife, it is not surprising that traits characterizing multi-host and specialist parasites have been of much interest. A study of parasites in wild primates found that nearly half of all primate viruses infected multiple host orders, while nearly half of all primate helminths were species-specific (Pedersen *et al.* 2005) From this, the authors suggest that parasites with rapid generation times and high rates of mutation (*e.g.,* viruses) are more likely to be generalists, while parasites with slow generation times and complex life histories (*e.g.,* helminths) are more likely to be specialists (Pedersen *et al.* 2005).

Host specificity is also affected by the mode of parasite transmission. Parasites can be transmitted directly (*e.g.,* bites, scratches, touching, mating), environmentally (*e.g.,* fomites, contact with contaminated substrates), or through vectors (*e.g.,* biting arthropods). Parasites that are directly transmitted have limited opportunity to infect new species, because it is uncommon for one host species to interact closely with another (Woolhouse *et al.* 2001). In contrast,

parasites that are indirectly transmitted (via the environment or vectors) may have more frequent exposure to alternative hosts and thus tend towards generalism. Therefore, vector transmitted parasites are expected to have broad host ranges to match the breadth of species that a vector bites (Woolhouse *et al.* 2001; Pedersen *et al.* 2005).

1.3.4 Predicting Transmission in Communities

Although considerable attention has focused on understanding the impact of multi-host parasites and the traits that are common to them, very little research has examined what determines the identity of infected species. Two broad hypotheses have been proposed (Page 2003; Davies & Pedersen 2008). The first suggests that multi-host parasites infect closely related species. This may be because closely related hosts inherit parasites through common descent, or because parasites can colonize hosts that are similar in physiology and life history to their current host. The second suggests that multi-host parasites infect geographically overlapping species. If adaptation to a new host can occur rapidly, then exposure to hosts is more important than physiological similarity. Therefore, species that occupy similar niches or overlap in territory will share parasites (Antonovics *et al.* 2002; Davies & Pedersen 2008). In a study that examined these two hypotheses in wild primates, phylogenetic relatedness was the best overall predictor of parasite sharing. Indeed, the authors found that humans were four times more likely to share pathogens with a close primate ancestor, chimpanzees, than with a more distant primate ancestor, colobus monkeys (Davies & Pedersen 2008). Since this study, a number of host-parasite systems have found that parasites are more commonly shared between close relatives (Krasnov *et al.* 2010; Poulin 2010; Streicker *et al.* 2010), although a recent study shows that while phylogenetic relationships are important, parasite sharing among hosts can be more fully explained by including host ecology (Cooper *et al.* 2012). Therefore, patterns of host specificity may be most accurately predicted by merging both possible explanations (phylogeny and geography) into a framework that considers the distributional dynamics of hosts.

1.3.5 Thesis Predictions Based on Theory

My thesis empirically examines theoretical predictions of host specificity and transmission in two taxonomic groups of parasites: soil-transmitted helminths (nodule worm *Oesophagostomum* and whipworm *Trichuris*) and vector-transmitted protozoans (blood parasites *Plasmodium* and

Hepatocystis). Helminths have slow generation times and complex physiology that may prevent adaptation to multiple hosts. Therefore, I predicted that these parasites would infect few, closely related, hosts (*i.e.*, they would be host specialists). In contrast, protozoan blood parasites have rapid generation times and are vector-borne. Therefore, I predicted that these parasites would infect multiple hosts (*i.e.*, they would be host generalists).

As per Davies and Pedersen (2008) and Cooper *et al.* (2012), I expected that patterns of generalist parasite infection would follow the phylogenetic relatedness of hosts. Since I examined a community of primates with a gradient of phylogenetic distances between species, I predicted that closely related hosts would have the same or similar parasites, while more distantly related hosts would have distinct lineages or species of parasites. For example, humans and their closest relatives, chimpanzees, would share more parasites than they would with their more distant relatives, colobus monkeys.

1.4 Neglected Tropical Diseases

The parasites investigated in my thesis are part of a sub-group of infectious diseases called Neglected Tropical Diseases (NTDs), which affect ~1.5 billion people globally (Fenwick 2012). This group of diseases is comprised of a number of biologically disparate parasites, including bacteria, protozoa, and helminths. They are united by three shared characteristics. First, as their name implies, NTDs are typically endemic to tropical and sub-tropical regions. Second, NTDs predominate in low-income or impoverished populations. Most NTDs are preventable through access to clean water and sanitation. Others are eradicable, if already existing medications are made broadly accessible (Feasey *et al.* 2010). Finally, NTDs are largely overlooked in research and policy, at least partly because their association with poverty negates profitable exploration (Yamey & Hotez 2007).

The majority of NTDs do not typically result in death. Many, however, including leprosy, onchocerciasis (river blindness) and leishmaniasis cause deformations that handicap infected individuals. Others can reduce productivity through cognitive or physical sluggishness, which is associated with diminished labour outputs (Hotez *et al.* 2008; Hotez *et al.* 2009). So, despite

limited mortality, the burden of NTDs is thought to be far greater than current metrics (such as Disability Adjusted Life Years) estimate (Engels & Savioli 2006).

My thesis research focuses on two groups of NTDs: soil-transmitted helminths and malaria (or malaria-like) parasites. In addition to the theoretical contributions my research may have, there are also practical benefits to understanding the transmission and consequences of NTDs in an ecological community. Although this field of research has received very little attention to-date, the increasing proximity between humans and wildlife occurring globally means that such information will likely be important for future public health and conservation policies.

1.4.1 Soil-Transmitted Helminths

Soil-transmitted helminths (STHs) are a group of parasitic nematode worms that cause infection in ~1 billion people globally (Bethony *et al.* 2006). These worms are transmitted environmentally through contact with soil, food, or water that is contaminated with parasitic eggs or larvae. The greatest burden of infection is suffered by school-aged children, where these parasites are shown to cause stunting, reduced academic performance, and diminished future wage-earning capacity (Miguel & Kremer 2004; Bethony *et al.* 2006; Bleakley 2007). Individuals with STHs are also frequently co-infected with malaria or HIV/AIDS. Importantly, helminth infections are known to exacerbate the symptoms and progression of both these lethal diseases (Druilhe *et al.* 2005; Borkow & Bentwich 2006).

Three groups of STHs are considered major worldwide pathogens: roundworms (*Ascaris lumbricoides*), whipworms (*Trichuris trichiura*), and hookworms (*Necator americanus* and *Ancylostoma duodenale*). Nodule worms (*Oesophagostomum bifurcum*) are restricted in range to foci in West Africa, and are therefore considered important locally.

Among the parasites of wild primates, helminths represent the nearly half (43%) of described diversity. This is in contrast to humans, where helminths comprise only 20% of parasites (Pedersen *et al.* 2005). A number of STH species are known to infect primates, and *Trichuris*, *Oesophagostomum*, *Strongyloides*, and other strongyle nematodes are most commonly documented on the African continent (Munene *et al.* 1998; Hahn *et al.* 2003; Gillespie *et al.*

2004, 2005b; Weyher *et al.* 2006; Teichroeb *et al.* 2009). I focused on *Trichuris* and *Oesophagostomum* in my research, since these are the two most commonly found gastrointestinal helminths in the primate community under investigation.

1.4.2 Blood Parasites

Malaria, caused by the protozoan *Plasmodium* spp., is a vector-borne disease that currently afflicts more than 216 million people worldwide (W.H.O. 2011). In 2010, the World Health Organization (W.H.O.) estimated that 655,000 deaths were caused by malaria. However, because this estimate is based on passive reporting by nations, the actual number of deaths caused by malaria may be 50-200% higher (Snow *et al.* 2005). The most susceptible demographic to infection is African children; an African child is estimated to die every minute from this disease (W.H.O. 2011). Four species commonly infect humans: *P. falciparum*, *P. vivax*, *P. ovale*, and *P. malariae* (Das Gupta 1938; Contacos *et al.* 1963; Deane *et al.* 1966). *Plasmodium falciparum* is responsible for the greatest morbidity and mortality in Africa (W.H.O. 2011).

Twenty-six species of malaria have been found to infect wild populations of primates (Coatney *et al.* 2003). Notably, five of these species are transmissible to humans: *P. knowlesi*, *P. cynomolgi*, *P. brasilianum*, *P. simium* and *P. inui* (Knowles & Das Gupta 1932). Humans have also been infected with *P. rodhaini* and *P. schwetzi*, species that commonly infect African apes, although the risk of natural transmission to humans has not been determined (Coatney *et al.* 2003).

A second genera of the Plasmodiidae family is a malaria-like protozoan that also infects primates, *Hepatocystis.* To date, *Hepatocystis kochi, H. simiae, H. bouzellezi*, and *H. cercopitheci* are known to infect African primates. *Hepatocystis* is similar to *Plasmodium* in most respects, although *Hepatocystis* is transmitted by biting midges of the genus *Culicoides* instead of *Anopheles* mosquitoes. *Hepatocystis* is thought to be asymptomatic, although studies examining the long-term effects of infection on host fitness, as well as the parasite's transmissibility to human populations, have not been conducted. I considered both *Plasmodium* and *Hepatocystis* in my research, since the transmission dynamics of these within natural communities remains uncharacterized.

1.5 Primates as Hosts

My thesis research uses primates as hosts to answer questions about host-parasite interactions. Primates make interesting hosts for four important reasons. First, primates are host to an exceptional diversity of parasites from all taxonomic groups, including viruses, bacteria, protozoa, and helminths (Nunn *et al.* 2003). Primates may be favourable hosts for parasites because of their socio-ecology – most species are highly social and gregarious, which is shown to be positively correlated with greater diversity and abundance of parasites (Côté & Poulin 1995; Arneberg 2002; Nunn *et al.* 2003; Nunn & Altizer 2006). Second, perhaps because of their considerable parasite burdens, primates employ "anti-parasite" behaviours to combat infection (Møller *et al.* 2001). For example, all species groom, which serves the hygienic function of removing ectoparasites (*e.g.*, lice and fleas), as well as parasite infective stages like larvae and vectors (Nunn & Altizer 2006; MacIntosh *et al.* 2012). In addition, all major lineages of primate are documented to self-medicate (*i.e.*, deliberately consume foods to remove or alleviate infection; see Nunn & Altizer (2006) for review).

Primates are also ecologically important because of their ability to modify habitat through their feeding strategies (*i.e.,* they are ecosystem engineers). Specifically, primates are important seed dispersers, and can deplete and kill preferred food trees, which fundamentally alters tree community structure (Chapman *et al.* 2013). Since the majority of primates are declining in both distribution and abundance, understanding potential risk factors involved in primate declines (such as parasitic disease) could benefit conservation and shed light on future compositional changes in ecosystems that include primates.

Finally, because humans are part of the Primate order, understanding primate disease transmission can directly benefit public health. A number of humanity's most lethal diseases have emerged from wild primates, including HIV/AIDS, Ebola, and malaria (Gao *et al.* 1999; Liu *et al.* 2010). Disease transmission is also not unidirectional; wild primates have suffered a number of epidemics resulting from human disease. For example, widespread mortality in endangered great ape populations have resulted from polio and tuberculosis (Chapman *et al.* 2005a). The probable reason for a disproportionate number of transmission events between human and non-human primates is that recent common descent makes us physiologically and

immunologically similar. Indeed, this similarity is the motivating reason for using primates in experimental and laboratory medicine. Given this, it is perhaps not surprising that some studies have identified wild primates as one of the most significant sources of human parasitic diseases (Wolfe *et al.* 1998; Wolfe *et al.* 2007; Pedersen & Davies 2009).

1.6 Kibale National Park

My thesis focused on host-parasite interactions in Kibale National Park (hereafter Kibale), which is a 795 km^2 protected area in western Uganda. Kibale is located just North of the equator (0˚13' - 0˚41' N and 30˚19' - 30˚32' E) and near the foothills of the Ruwenzori Mountains that border Uganda and the Democratic Republic of the Congo (Figure 1.1). Kibale is classified as moist evergreen and semi-deciduous tropical rainforest, although the land cover of Kibale is a mosaic of unlogged and regenerating forest, shrubs, grasslands, and wetlands (Jacob *et al.* 2014).

1.6.1 History

In 1932, Kibale was gazetted a Forest Reserve under the management of the Uganda Forest Department, with the explicit purpose of providing timber. Commercial, mechanized logging began in the early 1940s in the North and center of the reserve, and ceased in the early 1970s. During civil unrest in the 1970s and 80s, forest management was reduced or arrested altogether throughout Uganda (Jacob 2014). Following the restoration of democratic rule, Kibale was afforded National Park status under management of the Uganda Wildlife Authority in 1993 (Struhsaker 1997). In the years since logging cessation, variation in logging intensity between forestry blocks ('compartments') created a system to study gradients in the effects of disturbance on wildlife and forest recovery (Struhsaker *et al.* 1996; Chapman & Chapman 1997; Olupot *et al.* 1997; Struhsaker 1997; Chapman *et al.* 2000; Gillespie *et al.* 2005a).

1.6.2 Kanyawara

My research was conducted in the Kanyawara region of Kibale, which harbours an active biological field station run through Makerere University. Prior to Kibale becoming a national park, the Kanyawara region was divided into four forestry compartments that underwent differing logging intensities. The K15 and K31 forestry compartments are estimated to have lost 50% of the forest canopy to logging and incidental damage. The K14 forestry compartment is

estimated to have lost 25% of the canopy, and the K30 compartment was not commercially harvested. Data for all four of my thesis chapters were collected from these four compartments, which have been protected from hunting and logging since the 1970s (Chapman & Chapman 1997; Struhsaker 1997).

Kanyawara is highly biodiverse, but is especially renowned for the species richness and abundance of primates. The diurnal primate community is composed of eight species, including chimpanzees (*Pan troglodytes* ssp. *schweinfurthii*), grey-cheeked mangabeys (*Lophocebus albigena*), olive baboons (*Papio anubis*), blue monkeys (*Cercopithecus mitis*), red-tailed guenons (*Cercopithecus ascanius*), l'hoest's monkeys (*Cercopithecus l'hoesti*), black-and-white colobus (*Colobus guereza*), and red colobus (*Procolobus rufomitratus*). The International Union for Conservation of Nature (IUCN) lists two of these species as endangered: the eastern chimpanzee, and the red colobus. Indeed, Kibale is believed to harbour the largest populations of chimpanzees in East Africa and the only viable population of red colobus (*P. r.* ssp. *tephrosceles*) (Plumptre *et al.* 2003; Struhsaker 2005). The Kanyawara primates are well-studied; research in Kanyawara has occurred for nearly 40 years, there are long-term data available for many species, and most are habituated to human presence (Chapman *et al.* 2000).

1.6.3 Human Settlement

Prior to human settlement, Kibale existed as part of a continuous forest in the region. Human forest clearance is estimated to have begun 4,800 years ago (Hamilton *et al.* 1986) and has continued since. Today, Kibale is an isolated fragment surrounded by one of the densest congregations of human settlements in East Africa, with ~600 people/km^2 living outside of the Kanyawara research site (Mackenzie & Hartter 2013). This population is growing rapidly (by 3.2% per year), and is very poor (40% living below the global poverty line; Uganda Bureau of Statistics 2009; FAO 2012; Jacob 2014). Rural settlements are arranged into villages of ~100 households (Mackenzie & Hartter 2013). The majority of individuals are subsistence farmers whose livelihoods are tied to agriculture; only 22% of adults in this population are employed (Mackenzie 2012).

1.6.4 Interactions between People and Primates

The conservation policy implemented to protect Kibale involves strict exclusion of local people (with the exception of groups with resource access agreements) and enforcement of the park boundary. Despite this, people illegally enter the forest, and primates often leave it. Spatial interactions such as these create the conditions for cross-species disease transmission to occur.

1.6.4.1 People in the Protected Area

Aside from researchers, employees, tourists, and select local groups with resource access agreements, people are prohibited from entering Kibale. Households directly adjacent to the Kibale boundary therefore face a barrier to extracting resources that may result in smaller resource pools than those of households residing further away (Hartter 2010). Resource-limited individuals/households may therefore take to illegally extracting resources from the protected area. There are many resources within Kibale that are desired by the local population, including wood (for cooking, heating, and making bricks), craft materials, medicinal plants, area to graze livestock or keep beehives, and to a lesser extent, protein from bushmeat (Naughton *et al.* 2006; Mackenzie & Hartter 2013). In a study that quantified patterns of illegal extraction along the Kibale boundary, the number of extracted trees and illegal entry trails were highest outside the Kanyawara study area (Mackenzie *et al.* 2012).

1.6.4.2 Primates outside the Protected Area

A number of wildlife species in Kibale exit the national park to raid food from adjacent farms. The most devastating crop raiders are elephants, because they can destroy a farmer's entire yield in a single night. Primates more frequently raid crops however. The most common offenders are baboons, red-tailed guenons and chimpanzees (Naughton 1998). A recent study used household surveys to determine the financial and social costs of crop raiding animals. Results indicate that the majority of crop raiding events occur within 50 m of the protected area boundary. In addition, residents living adjacent to the Kanyawara study area reported being the most troubled by crop raiding animals relative to other areas around the park. Interestingly, households experiencing crop raiding also reported higher rates of human and livestock disease, which suggests that crop raiding may be directly or indirectly associated with disease transmission between wild primates and humans (Mackenzie & Ahabyona 2012).

1.6.5 Suitability of Kibale for My Research

There are six reasons why Kibale was an ideal study site for my research:

1. The primates of the Kanyawara region of Kibale were mostly habituated to human observers. This made it feasible to collect samples entire diurnal primate community.

2. The primate community is composed of eight species with varying degrees of niche and phylogenetic relatedness. This allowed me to test whether the transmission of generalist parasites is best predicted by host phylogeny or geographic overlap.

3. The gastrointestinal parasites present in the diurnal primate community were previously identified and characterized to genus level (Gillespie *et al.* 2004, 2005b; Bezjian *et al.* 2008). This information allowed me to focus on developing molecular methods and models to test predictions on parasites that I already knew were present.

4. Kibale is located within the Albertine Rift, considered one of the world's most threatened biodiversity hotspots (Plumptre *et al.* 2007) (Figure 1.2). Importantly, the biodiversity of the region makes it likely to harbour undiscovered or unclassified parasites. This is supported by previous research in Kibale, which has discovered a number of new or divergent viruses in the diurnal primate community (Goldberg *et al.* 2008a; Goldberg *et al.* 2009; Lauck *et al.* 2011; Lauck *et al.* 2012; Sibley *et al.* 2014).

5. The Kanyawara study area experiences some of the most intense and frequent interaction between primates and local people, which makes it an ideal location to test for transmission of parasites between wild primates and humans.

6. Previous research has suggested that areas at greatest risk of parasitic disease emergence will be tropical forest communities with high levels of biodiversity and exterior agricultural settlements with high population density (Wolfe *et al.* 1998). Kibale satisfies these requirements, and in addition, is situated in a region of the world predicted to be at greatest risk of disease emergence (Pedersen & Davies 2009).

1.7 Thesis Outline

The overarching objective of my thesis was to conduct an in-depth study of host-parasite interactions that would determine what dictates parasite transmission, as well as consequences of these parasites within their hosts. To meet these objectives, chapters 2, 3 and 4 examined the host range and transmission of three separate parasites with different traits and life histories. This allowed me to determine whether the transmission dynamics I observed agreed or contrasted with my predictions based on host-parasite theory. Chapter 5 examined the consequences of typically non-lethal parasites on hosts, which allowed me to determine whether the non-lethal parasites that were examined throughout my thesis may still impose consequences on their hosts.

1.7.1 Chapters 2 & 3

In Chapters 2 and 3, I characterized the host range and transmission of two soil transmitted helminths: *Oesophagostomum* (nodule worm) and *Trichuris* (whipworm), respectively. I collected samples from all eight diurnal primates in the community, and used these for both chapters. This allowed me to directly compare both chapters, because parasites were identified from not only the same community, but from the same individuals (see Appendix A).

In Chapter 2, I investigated the host range and transmission dynamics of the nodule worm, *Oesophagostomum*. I collected fecal samples from all species of diurnal primate in Kibale (eight in total), as well as samples from people living adjacent to the study area. I used molecular methods (parasite DNA extraction, PCR amplification and sequencing) to identify lineages of *Oesophagostomum* circulating in the community and assess the range of primates each lineage infected.

I followed a similar procedure in Chapter 3, but here I focused on the whipworm, *Trichuris*. I used the same samples as in Chapter 2, which allowed me to directly compare the transmission of *Oesophagostomum* to *Trichuris* within an identical sample set (see Appendix A). Since these two soil transmitted helminths have similar life history and mode of transmission, I expected to find similar patterns of host range in each.

Chapter 2 and 3 addressed four questions:

What is the diversity of soil transmitted helminths (Oesophagostomum and Trichuris) within wild primate communities?

Are soil transmitted helminths host specialists (i.e., they infect a narrow range of hosts)?

How do host ranges differ between soil transmitted helminths with similar mode of transmission and life history?

What dictates host range – phylogenetic relatedness or geographic overlap between hosts?

1.7.2 Chapter 4

In Chapter 4, I characterized the host range and transmission of *Plasmodium* and *Hepatocystis*. I collected human blood samples and used primate blood samples that were collected as part of a larger initiative. Because of the invasive nature of blood sample collection, we were unable to collect blood from chimpanzees (protected from invasive sampling) or l'hoest monkeys (not sufficiently habituated to allow darting). Using molecular methods, we screened for both *Plasmodium* and *Hepatocystis*. Surprisingly, we did not detect *Plasmodium* in any primates sampled, despite its reported presence in Kanyawara chimpanzees (Krief *et al.* 2010a), and other natural primate populations (Coatney *et al.* 2003), as well as its demonstrated ability to infect African monkeys (Prugnolle *et al.* 2011). In addition, we did not detect *Hepatocystis* in humans, although this is unsurprising (it is not believed to be hazardous for cross-transmission to humans) (Seethamchai *et al.* 2008). As a result, this chapter was divided into two manuscripts – one that investigates the transmission of *Hepatocystis* within the diurnal Kibale monkey community (Part I; Thurber & Ghai *et al.* 2013), and one that describes the issue of malaria misdiagnosis in rural Africa (Part II). Overall, this chapter addresses five questions:

What is the diversity of blood parasites (Hepatocystis and Plasmodium) within wild primate communities?

Are blood parasites host generalists (i.e., they infect a broad range of hosts)?

How frequently do blood parasites cross-transmit to other host species?

How does the host range of blood parasites in this community compare to the host range of soil transmitted helminths?

How often are patients with fever misdiagnosed for malaria?

1.7.3 Chapter 5

Chapter 5 investigated the relationship between parasitic infection and host behaviour, using a generalist parasite that does not normally result in mortality (the whipworm, genus *Trichuris*). I used long-term data from a well-studied red colobus group, paired with fecal samples that I screened for parasites and detailed focal data that I collected from the same group. I identified that whipworm-positive individuals showed signs of lethargy, since they increased resting and decreased energetic behaviours. I also found evidence for self-medication, which suggests active defense against parasitism. This chapter addresses three questions:

How do non-lethal parasites affect primate behaviour?

Do primates actively defend against parasitic infection?

What consequences might non-lethal parasites have on primate hosts?

1.8 Ethics Statement

All research included in this thesis was conducted following approval of the Uganda National Council for Science and Technology, the Uganda Wildlife Authority, the McGill Animal Care and Use Committee, and both McGill and University of Wisconsin-Madison Research Ethics and Compliance Boards.

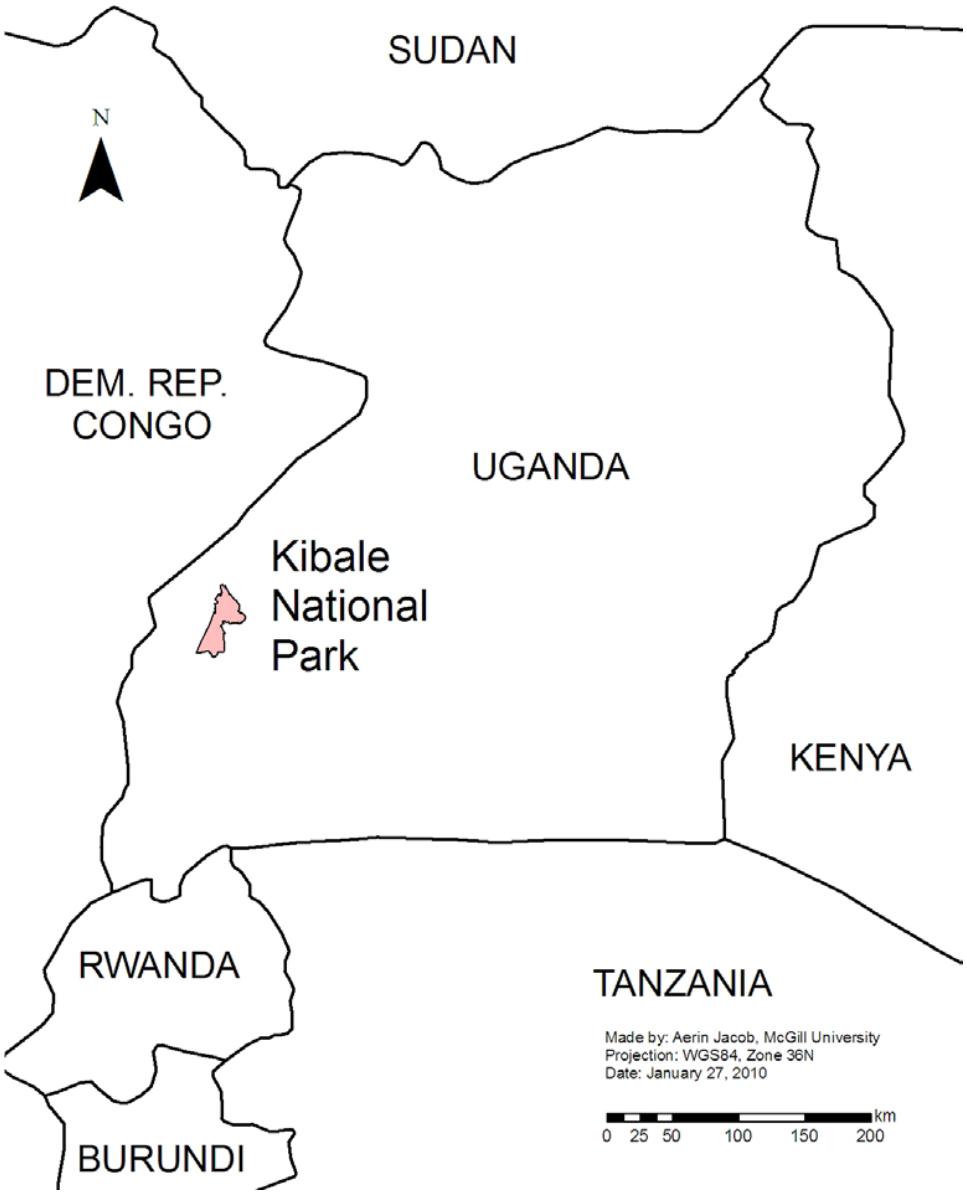
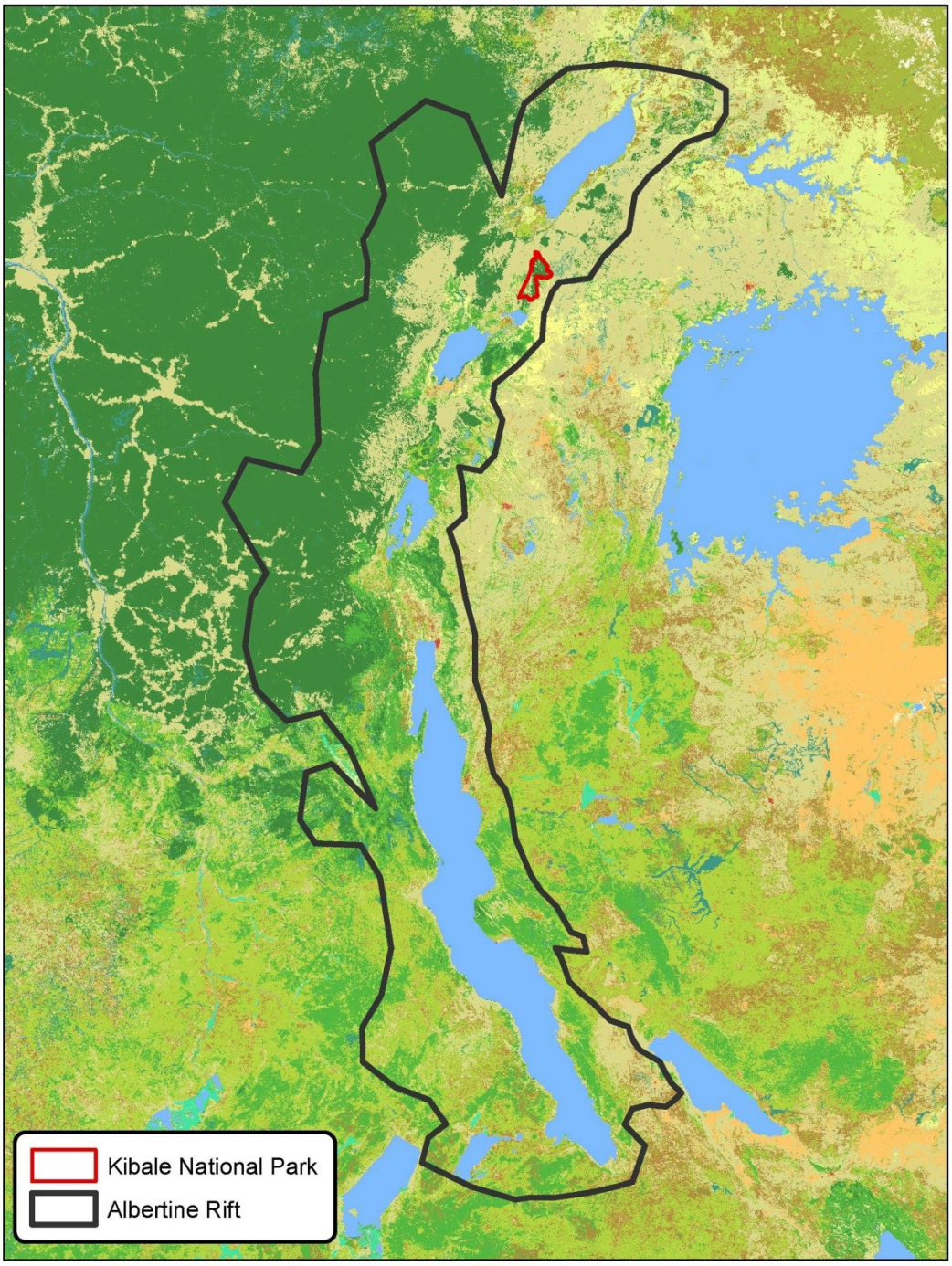


Figure 1.1: Kibale National Park. Location, size and shape of Kibale National Park (0° 13'- 0° 41' N and 30° 19' – 30° 32' E) within Uganda.



Created by Johanna Bleecker, Chapman Lab 2012. Albertine Rift outline provided by Wildlife Conservation Society. Land use imagery courtesy of the ESA GlobCover 2009 Project.

Figure 1.2: Kibale National Park within the Albertine Rift.

CHAPTER 2

Nodule worm infection in humans and wild primates in Uganda: cryptic species in a newly identified region of human transmission

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2.1 Abstract

2.1.1 Background

Soil-transmitted helminths (STHs) are a major health concern in tropical and sub-tropical countries. *Oesophagostomum* infection is considered endemic to West Africa but has also been identified in Uganda, East Africa, among primates (including humans). However, the taxonomy and ecology of *Oesophagostomum* in Uganda have not been studied, except for in chimpanzees (*Pan troglodytes*), which are infected by both *O. bifurcum* and *O. stephanostomum.*

2.1.2 Methods and Findings

We studied *Oesophagostomum* in Uganda in a community of non-human primates that live in close proximity to humans. Prevalence estimates based on microscopy were lower than those based on polymerase chain reaction (PCR), indicating greater sensitivity of PCR. Prevalence varied among host species, with humans and red colobus (*Procolobus rufomitratus*) infected at lowest frequency (25 and 41% prevalence by PCR, respectively), and chimpanzees, olive baboons (*Papio anubis*), and l'hoest monkeys (*Cercopithecus lhoesti*) infected at highest frequency (100% prevalence by PCR in all three species). Phylogenetic regression showed that primates travelling further and in smaller groups are at greatest risk of infection. Molecular phylogenetic analyses revealed three cryptic clades of *Oesophagostomum* that were not distinguishable based on morphological characterization of their eggs. Of these, the clade with the greatest host range has not previously been described genetically. This novel clade infects humans, as well as five other species of primates.

2.1.3 Conclusions

Multiple cryptic forms of *Oesophagostomum* circulate in the people and primates of western Uganda, and parasite clades differ in host range and cross-species transmission potential. Our results expand knowledge about human *Oesophagostomum* infection beyond the West African countries of Togo and Ghana, where the parasite is a known public health concern. *Oesophagostomum* infection in humans may be common throughout sub-Saharan Africa, and the transmission of this neglected STH among primates, including zoonotic transmission, may vary among host communities depending on their location, composition, and ecology.

2.2 Introduction

Soil-transmitted helminths (STHs) are parasitic nematodes that cause infection *via* eggs and larvae, which are shed in feces and persist in the soils of tropical and sub-tropical countries (Bethony *et al.* 2006). STHs infect over one billion people worldwide (W.H.O. 2004) and may cause a combined disease burden as substantial as that caused by malaria or tuberculosis (Chan 1997). Nevertheless, these parasites are largely neglected in research, perhaps in part because the diseases they cause are suffered by the world's most impoverished populations (Bethony *et al.* 2006). Although roundworms (*Ascaris lumbricoides*), hookworms (*Necator americanus* and *Ancylostoma duodenale*) and whipworms (*Trichuris trichiura*) are of global importance, other "lesser" parasites are localized to specific regions (Bethony *et al.* 2006). This includes *Oesophagostomum* spp., a genus of nodule-causing worms with L3 larvae that are infective via ingestion after 4-7 days (Chabaud & Larivière 1958; Crestian & Crespeau 1975; Polderman & Blotkamp 1995; de Gruijter *et al.* 2004). The human burden of *Oesophagostomum* infection is considered localized to West Africa, specifically the countries of Togo and Ghana (Polderman *et al.* 1991; Krepel 1994; Polderman & Blotkamp 1995).

A variety of mammals, including pigs, ruminants (Skrjabin *et al.* 1961; Stewart & Gasbarre 1989), and non-human primates are frequently parasitized by *Oesophagostomum*. Infections in wild primates appear to be asymptomatic; clinical signs and mortality due to *Oesophagostomum* have only been recorded in captivity (Stewart & Gasbarre 1989; Krief *et al.* 2008). Eight species of *Oesophagostomum* have been recorded in wild primates, of which the three most common, *O. bifurcum*, *O. stephanostomum*, and *O. aculeatum*, are able to infect humans (Chabaud & Larivière 1958; Skrjabin *et al.* 1961; Blotkamp *et al.* 1993; Polderman & Blotkamp 1995). Of these, *O. bifurcum* appears to be the only species to regularly parasitize humans, with human infections by other species considered incidental (Krepel 1994; Polderman & Blotkamp 1995). In Togo and Ghana, the majority of human *Oesophagostomum* cases occur within endemic foci (Polderman *et al.* 1991; Polderman & Blotkamp 1995) and affect 20% and 90% of the population, respectively, with prevalence highest in rural areas (Krepel *et al.* 1992; Storey *et al.* 2000; Krief *et al.* 2008). The only known species to cause infection within these countries is *O. bifurcum*, which also infects the region's non-human primates, including patas monkeys (*Erythrocebus patas*), mona monkeys (*Cercopithecus mona*), and olive baboons (*Papio anubis*)

(Stewart & Gasbarre 1989; de Gruijter *et al.* 2004; van Lieshout *et al.* 2005). However, previous research has indicated that *O. bifurcum* is not commonly transmitted among primate species (including humans) because different parasite variants within the species are adapted to specific hosts (de Gruijter *et al.* 2004; de Gruijter *et al.* 2005; van Lieshout *et al.* 2005).

In Uganda, a number of primate species harbor *Oesophagostomum,* as evidenced by microscopic detection of eggs in feces. These include members of the primate subfamilies Cercopithecinae and Colobinae, as well as chimpanzees (*Pan trogolodytes*) (Gillespie *et al.* 2004, 2005b; Krief *et al.* 2005b). There have also been reports of oesophagostomiasis in human patients in Uganda, although no such reports, to our knowledge, have been published since the 1980s (Anthony & McAdam 1972; Polderman & Blotkamp 1995), perhaps due to under-reporting or improvements in treatment. With the exception of chimpanzees, which are infected with both *O. bifurcum* and *O. stephanostomum* (Krief *et al.* 2010b), the species of *Oesophagostomum* infecting Ugandan primates and humans remains unknown.

In this study, we examined the diversity of *Oesophagostomum* within the primate community of Kibale National Park, Uganda, using a combination of microscopic and molecular methods. Species-specific identification of eggs by microscopy alone is difficult, because eggs are similar morphologically to other STHs, including hookworms, *Trichostrongylus* spp., and the "false hookworm" *Ternidens deminutus* (Goldsmid 1968; Blotkamp *et al.* 1993; Krepel 1994; Polderman & Blotkamp 1995; Gasser *et al.* 2006). In other studies, coproculture of L3 larvae or necropsy to isolate adult worms have been used to identify these parasites to species (Blotkamp *et al.* 1993; Gasser *et al.* 1999). Here, we used molecular methods to detect *Oesophagostomum* DNA directly in feces; such methods have proven informative for other similar studies (Gasser *et al.* 1993; Gasser *et al.* 2009). In addition, we used phylogenetic comparative methods to ascertain whether primate host traits explain variation in prevalence of *Oesophagostomum* infection among host species. Our sampling and analyses included nearby human populations to assess whether *Oesophagostomum* is a public health concern in the region, as well the parasite's local propensity for zoonotic transmission.

2.3 Methods

2.3.1 Study Site and Sample Collection

Kibale National Park (0°13'-0°41'N, 30°19'-30°32' E) is a 795 km^2 semi-deciduous protected area in western Uganda. Primate research has occurred in Kibale for over four decades, focusing on chimpanzees and red colobus monkeys (*Procolobus rufomitratus*) (Chapman *et al.* 2005b; Watts 2012). As a result, a number of primate groups are habituated to human presence, and many individuals are recognizable based on a combination of physical attributes and collars affixed as part of a larger project on primate health and conservation (Goldberg *et al.* 2012).

Samples from monkeys in the Kanyawara area of Kibale National Park were collected from red-tailed guenons (*Cercopithecus ascanius*), blue monkeys (*Cercopithecus mitis*), l'hoest monkeys (*Cercopithecus lhoesti*), grey-cheeked mangabeys (*Lophocebus albigena*), olive baboons (*Papio anubis*), red colobus, and black-and-white colobus (*Colobus guereza*) (Figure 2.1). Chimpanzee samples were collected from Kanyanchu, an area that has a habituated chimpanzee community as a result of tourism (Figure 2.1). All samples were collected non-invasively immediately after defecation and placed into sterile tubes. Date, location, species, age and sex category, and social group membership were recorded. Human samples were collected after the receipt of Institutional Review Board-approved informed consent following World Health Organisation protocols. Samples collection occurred in three villages: Ibura, Kanyansohera, and Kasojo, which are less than 5 km from the border of the park (Figure 2.1). Individuals between the ages of 2 and 70 were suitable participants of this study. Consenting participants were given instructions on how to collect the sample, which was then retrieved for processing within a day.

Samples were subjected to a modified ethyl acetate concentration method, recommended in the approved guidelines of the Clinical and Laboratory Standards Institute for the identification of intestinal-tract parasites (Garcia *et al.* 2005; Greiner & McIntosh 2009). Concentration by sedimentation was performed in the field using one gram of undiluted feces without fixture in formalin, as formalin is a known inhibitor of the polymerase chain reaction (Young *et al.* 1979). All materials were sterilized prior to use, and care was exercised throughout the procedure to prevent contamination. Sediments were left uncapped for two hours after completion of the procedure to allow ethyl acetate that may otherwise inhibit polymerase chain reaction (PCR) to

volatilize. Sediments were then suspended in 2 mL RNALater nucleotide stabilization solution (Sigma-Aldrich, St. Louis, MO, USA) and frozen at -20°C until shipment to North America.

2.3.2 Microscopy

Thin smears from sedimented feces were used for microscopy (W.H.O. 1991). All eggs of the genus *Oesophagostomum* were identified at 10X objective magnification on a Leica DM2500 light microscope. Data were recorded on size, shape, color and internal contents of eggs. Images were captured at 40X objective magnification of all specimens using an Infinity1 CMOS digital microscope camera and Infinity Camera v.6.2.0 software (Lumenera Corporation, Ottawa, ON, Canada). Samples were considered negative after the entire sediment sample was scanned and no eggs were found. We note that while identification of *Oesophagostomum* eggs was based on a rigorous set of characteristics, this genus cannot easily be distinguished from hookworm infection by eggs alone. However, hookworms have not been found in previous surveys of the gastrointestinal parasites of this primate community (Gillespie *et al.* 2004, 2005b), suggesting that eggs identified with shared characteristics of both *Oesophagostomum* and hookworm were almost certainly *Oesophagostomum*.

2.3.3 Molecular Methods

DNA was extracted from 200 µL of sedimented feces using a ZR Fecal DNA MiniPrep™ Kit (Zymo Research Corporation, Irvine, CA, USA), following manufacturer protocols. External PCR was performed targeting the ribosomal internal transcribed spacer 2 gene using primers NC1 (5'-ACGTCTGGTTCAGGGTTGTT-3') and NC2 (5'-TTAGTTTCTTTTCCTCCGCT-3'), which generated products that ranged in size from 280 to 400bp, suggesting that, as expected, the primer set detected a number of parasitic helminths present in the samples (Gasser *et al.* 1993; Romstad *et al.* 1997). Subsequently, an internal, semi-nested PCR was performed using a newly designed *Oesophagostomum*-specific primer, OesophITS2-21 (5'-TGTRACACTGTTTGTCGA AC-3'). This primer was generated by aligning publicly available sequences of the *Oesophagostomum* internal transcribed spacer 2 gene (Newton *et al.* 1997; Romstad *et al.* 1997; Newton *et al.* 1998; Gasser *et al.* 1999; Yu *et al.* 2012). In total, eight species of *Oesophagostomum* were represented in the alignment. Other species of varying relatedness, including other members of the taxa Chabertiidae (*Chabertia ovina,* Accession No. JF680981;

Ternidens deminutus, Accession No. HM067975), Strongylidae (*Strongylus vulgaris* (Campbell *et al.* 1995)), and Strongylida (*Necator americanus* (Romstad *et al.* 1997), and *Ancylostoma duodenale* (Chilton & Gasser 1999)) were also included. Priming regions were selected to be identical among all species of *Oesophagostomum* but divergent from other genera. ITS2-21 was highly specific as confirmed by sequencing, since all PCR products matched *Oesophagostomum* despite the fact that a number of other parasites (including *Strongyloides* and *Trichuris*), were identified in the same samples during microscopic examination. Primer ITS2-21 was paired with NC2 to generate amplicons of predicted size 260 bp.

External PCR was performed in 25 µL volumes using the FailSafe System (Epicentre Biotenchnologies, Madison, WI, USA) with reactions containing 1X FailSafe PCR PreMix with Buffer C, 1 Unit of FailSafe Enyme Mix, 2.5 picomoles of each primer (NC1 and NC2), and 1 µL of template. Reactions were cycled in a Bio-Rad CFX96 platform (Bio-Rad Laboratories, Hercules, CA, USA) with the following temperature profile: 94°C for 1 min; 45 cycles of 94°C for 15 sec, 50°C for 30 sec, 72°C for 90 sec; and a final extension at 72°C for 10 min. Internal PCR was performed in 25 µL volumes using the DyNAzyme DNA Polymerase Kit (Thermo Scientific, Asheville, NC, USA) with reactions containing 0.5 Units of DyNAzyme I DNA Polymerase, 1X Buffer containing 1.5 mM $MgCl_2$, 2.5 picomoles of each primer (OesophITS2-21 and NC2), and 1 µL of template. Reactions were cycled with the following temperature profile: 95°C for 1 min; 45 cycles of 95°C for 15 sec, 55°C for 30 sec, 70°C for 90 sec; and a final extension at 70°C for 5 min. Amplicons were electrophoresed on 1% agarose gels stained with ethidium bromide, and purified from gels using the Zymoclean Gel DNA Recovery Kit (Zymo Research Corporation, Irvine, CA, USA) according to the manufacturer's instructions.
Products were Sanger sequenced in both directions using primers OesophITS2-21 and NC2 on ABI 3730xl DNA Analyzers (Applied Biosystems, Grand Island, NY, USA) at the University of Wisconsin-Madison Biotechnology Center DNA Sequencing Facility. Sequences were hand-edited and assembled using Sequencher v4.9 (Gene Codes Corporation, Ann Arbor, MI, USA) and all ambiguous bases were resolved by repeat PCR and re-sequencing, as described above. All new sequences were deposited in GenBank, under Accession Numbers KF250585 - KF250660.

2.3.4. Phylogenetic Analyses

Sequences were aligned using the computer program ClustalX (Larkin *et al.* 2007) with minor manual adjustment. Published reference sequences were included to determine putative species (AF136575, Y11733, AF136576) and as outgroups (HQ844232, Y11738, Y11735, Y10790, AJ006149), and were trimmed to the length of the newly generated sequences using Mesquite v.2.75 (Maddison & Maddison 2001). Trimmed sequences yielded the same tree topology as did untrimmed sequences (by neighbor-joining method; results not shown), suggesting that the amplified region was sufficient for taxonomic discrimination. Phylogenetic trees were reconstructed using maximum likelihood in MEGA v.5.05 (Tamura *et al.* 2011) and the Hasegawa-Kishino-Yano substitution model (Hasegawa *et al.* 1985). Phylogenetic support was assessed using 1,000 bootstrap replicates. To estimate *Oesophagostomum* genetic diversity, percent nucleotide-level sequence identity among sequences was calculated as the uncorrected pairwise proportion of nucleotide differences (p-distance) in MEGA v5.05 (Tamura *et al.* 2011).

2.3.5 Statistical Analysis

Diagnostic performance of microscopy *versus* PCR was estimated by calculating sensitivity (*i.e.*, true positive rate) and specificity (*i.e.*, true negative rate) using MedCalc v.12.5.0 (MedCalc Software, Ostend, Belgium). Prevalence of infection was calculated as the number of samples found to be positive for *Oesophagostomum* divided by the total number of samples collected, with 95% confidence intervals calculated using the modified Wald method (Agresti & Coull 1998). To determine whether prevalence differed among primate host species, a chi-square test was conducted in Quantitative Parasitology v3.0 (Rozsa *et al.* 2000). To explore variation in prevalence among hosts while controlling for their phylogenetic non-independence, a phylogenetic least squared regression (PGLS) was conducted in R (R-Development-Team 2008) using the ape (Paradis *et al.* 2004) and caper (Orme 2012) libraries. Prevalence of *Oesophagostomum* was selected as the dependent variable, and various primate life history traits as independent variables: terrestriality (predominantly terrestrial *versus* predominantly arboreal), maximum home range (Barton *et al.* 1992; Kaplin 2001; Snaith & Chapman 2008; Janmaat *et al.* 2009; Pebsworth *et al.* 2012; Watts 2012), maximum group size (Oates 1977; Barton *et al.* 1992; Olupot *et al.* 1994; Chapman & Chapman 2000a; Mitani *et al.* 2001; Snaith & Chapman 2008; Watts 2012), percentage time spent in polyspecific associations (Struhsaker 1981; Ham 1994),

average female body mass, and average daily travel distance (the latter was log transformed since the relationship was close to exponential) (Struhsaker 1981; Barton *et al.* 1992; Altmann *et al.* 1993; Kaplin 2001; Pontzer & Wrangham 2004). Humans were omitted from the PGLS analysis because many of these traits vary widely among human populations, making accurate estimations problematic.

To determine the degree to which each *Oesophagostomum* lineage (*i.e.*, taxonomic unit) identified by DNA sequencing was host restricted, we calculated the phylogenetic dispersion of infected hosts using the net relatedness index (NRI) in R (R-Development-Team 2008) using the ape (Paradis *et al.* 2004) and picante libraries. Mean pairwise distance (MPD) was weighted by the ratio of occurrence of each *Oesophagostomum* within each lineage, and compared to null expectation in 1000 randomly assembled communities. Results are reported as standard effects sizes, with positive values indicating phylogenetic evenness (*i.e.*, *Oesophagostomum* lineages infect a greater diversity of hosts than would be expected by chance), while negative values indicate phylogenetic clustering (*i.e.*, *Oesophagostomum* lineages are limited by host phylogeny in the range of hosts they can infect).

2.4 Results

A total of 318 fecal samples from primates, including humans, were collected (Table 2.1). Of these, 112 were identified as positive for *Oesophagostomum* by microscopy, making the community-wide prevalence of infection 35.2% (Table 2.1). All eggs identified through microscopy were similar in internal and external morphology in samples from all primate species (Figure 2.2). Eggs were 65-80 by 35-50 μm in size, which is consistent with previous results from this community (Gillespie *et al.* 2004, 2005b) (Figure 2.2).

PCR generated single, clear bands of expected size (260bp) in 222 samples, indicating positive detection of *Oesophagostomum*, for an overall prevalence of 69.8%. Assembled sequences overlapped 100% with published sequences and contained no insertions or deletions, making alignment unequivocal.

When PCR results were compared to microscopy, the overall sensitivity of PCR was 100% (95% CI 96.76-100), but specificity was only 47.5% (95% CI 40.47-54.65). Thus, PCR did not classify any microscopy-positive samples as negative but identified 109 microscopy-negative samples as positive.

Prevalence of *Oesophagostomum* infection (as determined by both microscopy and PCR) varied significantly among host species (chi-square, df=8, microscopy chi-square=54.31, p <0.0001; PCR chi-square= 112.2, p <0.0001). Both microscopy and PCR identified humans as having the lowest prevalence of infection (8.3% and 25%, respectively), followed by red colobus (17% and 41%, respectively). Chimpanzees, l'hoest monkeys, and olive baboons had the highest prevalence by both methods, with 100% prevalence by PCR in all three species (although sample sizes were low in some cases; Table 2.1).

PGLS indicated that terrestriality, maximum home range, maximum group size, percent of time spent in polyspecific associations, and average female body mass were not significant univariate predictors of *Oesophagostomum* prevalence (all p>0.05 from PGLS with lambda=ML; Table 2.2). However, log daily travel explained nearly 55% of the variation in prevalence among host species (P<0.05, R^2=0.546, from PGLS with the ML estimate of lambda=0). In a multivariate model, both group size and log daily travel were significant predictors of prevalence, with group size showing a negative relationship and log daily travel a positive relationship (Table 2.2). This two-predictor model including group size and daily travel explained over 75% of the variation in *Oesophagostomum* prevalence among species (model P <0.01, R^2= 0.7701; Table 2.3).

From 222 positive samples, 76 were selected for sequencing to represent as even a number of positive samples per host species as possible. All 76 sequences most closely matched published *Oesophagostomum* ITS-2 DNA sequences using the BLASTn tool on the National Centre for Biotechnology Information website. Phylogenetic analysis resolved these sequences into three clades (Figure 2.3). Clade 1 contained all 12 sequences from olive baboons, one sequence from l'hoest monkeys, one sequence from grey-cheeked mangabeys, three sequences from red colobus and one sequence from red-tailed guenons. These sequences were identical to published reference sequences for *O. bifurcum* (Romstad *et al.* 1997; Gasser *et al.* 1999). Five additional

sequences from l'hoest monkeys sorted into clade 1 and were 97.1% similar to this same *O. bifurcum* reference sequence. Clade 2 contained all eight sequences from chimpanzees, five sequences from blue monkeys, two sequences from black-and-white colobus, two sequences from grey-cheeked mangabeys, three sequences from red colobus, and twelve sequences from red-tailed guenons. All sequences in clade 2 were identical to an *O. stephanostomum* reference sequence (Gasser *et al.* 1999). Clade 3 was composed of two nearly identical branches (99.4% identity) that contained all six sequences from humans, as well as sequences from three blue monkeys, three black-and-white colobus, five grey-cheeked mangabeys, two red colobus, and two red-tailed guenons. These sequences were 92.4-93.0% and 93.0-93.6% similar to *O. bifurcum*, and *O. stephanostomum*, respectively, but were not identical to any published reference sequence.

Host species were phylogenetically clustered within *O. bifurcum* clade 1 (NRI= -1.76, $p < 0.05$). Clade 2 (*O. stephanostomum*) did not vary significantly from the null expectation (NRI= 0.86, $p = 0.75$). Clade 3 was phylogenetically even with respect to distribution of host species (NRI = 1.24, $p = 0.04$).

2.5 Discussion

Here we evaluated the prevalence of *Oesophagostomum* infection in wild primates and humans in western Uganda using both microscopy and PCR. Our results clearly show that prevalence varied significantly among host species. Humans had the lowest prevalence of infection likely because of avoidance behaviors such as sanitation practices (Slifko *et al.* 2000; Barreto *et al.* 2010) and because of the common use of antihelminthics in the region. Red colobus and black-and-white colobus also had comparatively low prevalence of infection, as found in previous studies (Nunn *et al.* 2003; Gillespie *et al.* 2005b; van Lieshout *et al.* 2005). This observation may reflect colobine gastrointestinal physiology, which is characterized by folivory and foregut fermentation (Davies 1994), and the associated regular ingestion of plant secondary compounds that may suppress infection by pathogenic organisms (Clauss 2003). Conversely, the high prevalence of infection in chimpanzees, olive baboons, and l'hoest monkeys may be a result of reduced physiological barriers to infection or increased susceptibility. To explain this interspecific variation in prevalence, we examined correlations between life history variables and

prevalence among host species. We found that two variables, daily travel distance and group size, explain over 75% of the variance in *Oesophagostomum* prevalence among host species. Surprisingly, body mass, the strongest predictor of helminth species richness elsewhere, was not significant here (Nunn *et al.* 2003).

Previous studies have argued that group-living animals with small home ranges are likely to suffer high intensities of infection due to frequent environmental re-exposure (Freeland 1976; Ezenwa 2003; Nunn & Altizer 2006). Our results indicate the opposite in the case of *Oesophagostomum*: smaller primate groups with large daily travel distances had higher prevalence. Animals with larger day ranges may encounter greater habitat variation (Poulin & Morand 2004), which may increase exposure to *Oesophagostomum* from environmental sources. In addition, previous research has implicated terrestriality as an important factor affecting the prevalence of trematode parasites in primates (Kooriyama *et al.* 2012). In our study, the three host species with highest *Oesophagostomum* prevalence (chimpanzees, olive baboons and l'hoest monkeys) were also the only three predominantly terrestrial species. Although this trend was not statistically significant, it is possible that terrestrial primates contact soil more frequently, and thus the infective stages of STHs.

Although group size was not a significant predictor of prevalence in univariate analyses, our multivariate analysis found smaller groups with large daily travel distances to be at greatest risk of infection. This finding contrasts with previous studies showing that increased intragroup contact increases exposure (Loehle 1995; Nunn & Altizer 2006). In Kibale, positive associations between group size and parasite richness have been documented for protozoan parasites in mangabeys (Freeland 1979). However parasite richness is not necessarily associated with prevalence. Small primate groups might maintain high intra-group infection rates for certain parasites if transmission within the group is frequent, thus maintaining high prevalence (as seen here) without correspondingly high parasite richness.

Our study detected substantial cryptic phylogenetic diversity in *Oesophagostomum* infecting Ugandan primates. Currently, the principal human *Oesophagostomum* species is considered to be *O. bifurcum* (Polderman & Blotkamp 1995), while other great apes harbor *O. stephanostomum*

(Crestian & Crespeau 1975; Krief *et al.* 2005b; Krief *et al.* 2008). Recently, however, feces screened from chimpanzees inhabiting a northern sector of Kibale were identified as positive for *O. bifurcum*, making this the first discovery of *O. bifurcum* in non-human apes. The same study identified chimpanzees also infected or co-infected with *O. stephanostomum* (Krief *et al.* 2010b). In our phylogenetic analysis, we identified both *O. bifurcum* and *O. stephanostomum* in the Kibale primate community. However, we found only *O. stephanostomum* in chimpanzees, although the possibility of undetected *O. bifurcum* co-infections cannot be ruled out. In addition, we identified a third *Oesophagostomum* lineage that did not cluster with any published sequence and thus may represent a previously uncharacterized taxon. It is possible that this new taxon has remained undetected in previous molecular investigations. Specifically, we examined the OB primer that has been used previously to identify *O. bifurcum* (Romstad *et al.* 1997) and conclude that it would probably not amplify our newly identified taxon due to mismatched bases at both the 5' and 3' ends of the primer. It is therefore possible that the new taxon we identified exists elsewhere (*e.g.* in Togo and Ghana) but has been not been detected or differentiated from other members of the genus. However, we caution that these inferences are based on a short region of a single gene, and that sequencing additional genes as well as morphological characterization of L3 larvae and adults will be necessary to confirm these findings. Nonetheless, our results suggest a heretofore unappreciated degree of hidden genetic diversity within this well-described genus of parasites that are known to infect humans.

Interestingly, all *Oesophagostomum* sequences recovered from humans clustered with the previously undescribed lineage 3, and not with the published *O. bifurcum* sequence from humans elsewhere in Africa (clade 1) (Romstad *et al.* 1997). In Ghana, geographic separation between humans and non-human primates infected with *Oesophagostomum*, despite apparently conducive environments for zoonotic transmission, motivated efforts to determine the host range of the parasite using molecular methods (Gasser *et al.* 2009). Genome-wide analysis tools (amplified fragment length polymorphism, random amplification of polymorphic DNA) suggested that *O. bifurcum* clustered into distinct groups by host species, thus indicating that zoonotic transmission may be uncommon (de Gruijter *et al.* 2004; de Gruijter *et al.* 2005). By contrast, in our study, no such geographic separation existed between human and non-human primate infections. We found that both humans and non-human primates were infected with the novel *Oesophagostomum* clade

3, which is phylogenetically over-dispersed compared to the other *Oesophagostomum* clades. While our conclusions await verification from more detailed examination of the entire *Oesophagostomum* genome, our results nevertheless suggest that this novel clade may be broadly transmissible among species of distantly related primate hosts, including humans. The Kibale ecosystem is known for its high degree of spatio-temporal overlap between humans and non-human primates and its ensuing high rates of transmission of diverse pathogens across primate species (Goldberg *et al.* 2007; Goldberg *et al.* 2008c; Johnston *et al.* 2010; Salyer *et al.* 2012). Our results provide further evidence for cross-species pathogen transmission between wild primates and humans in this region.

Our paired analyses applying both microscopy and PCR to the same samples indicate that traditional methods based on microscopy may significantly underestimate prevalence. Concentration methods followed by microscopic visualisation of eggs in thin smears are considered definitive diagnostic methods for confirming soil-transmitted helminth (STH) infection (Allen & Ridley 1970; Utzinger *et al.* 2008). Previous studies that have used fecal sedimentation and microscopy have reported an *Oesophagostomum* infection prevalence of between 2.8% and 10% in wild Ugandan primates (Gillespie *et al.* 2004, 2005b). These values are considerably lower than what we report here using molecular methods; however, our results parallel other studies that have assessed prevalence using molecular methods (van Lieshout *et al.* 2005; Krief *et al.* 2010b). Not surprisingly, we find that PCR is more sensitive than microscopy, perhaps because it can detect *Oesophagostomum* infection even when eggs are not present. For example, tissues or secretions shed by adult worms into the intestinal lumen would be detected by PCR, as would eggs that have hatched into L1 larva prior to fixation during the sedimentation procedure.

To our knowledge, ours is the first study in several decades to report human *Oesophagostomum* infection in Uganda, a country that is over 3,000 km from known foci of infection in West Africa (Polderman & Blotkamp 1995). Given that the prevalence of *Oesophagostomum* was 25% in our sample of people, we suspect that this parasitic infection occurs more commonly across sub-Saharan Africa than previously thought, and may be causing infections that are untreated or misdiagnosed. Our finding of a previously genetically uncharacterized lineage of

Oesophagostomum that may be transmitted among primate species underscores that the diversity (genetic and otherwise) of this parasite genus may be under-sampled in Africa. Further ecological studies of *Oesophagostomum* in Uganda and elsewhere are needed to quantify the degree of enzootic versus zoonotic transmission. Regardless of the outcome of such research, our results suggest that *Oesophagsotomum* should be considered a pathogen of concern beyond its accepted foci of infection in Togo and Ghana, and perhaps across all of equatorial Africa.

Table 2.1: **Prevalence of *Oesophagostomum* spp**. Prevalence of *Oesophagostomum* in nine primate host species (including humans) in and near Kibale National Park, Uganda, based on microscopy and PCR, where N represents the host sample size, which corresponds to the number of fecal samples collected.

		Number Positive		Prevalence (95% CI)	
Species	N	Microscopy	PCR	Microscopy	PCR
BM (Blue monkey)	33	10	24	30.3 (17-47)	72.7 (56-85)
BW (Black-and-white colobus)	37	8	21	21.6 (11-37)	56.8 (41-71)
CH (Chimpanzee)	30	18	30	60.0 (42-75)	100 (86-100)
GM (Gray-cheeked mangabey)	42	17	39	40.5 (27-56)	92.9 (80-98)
HU (Human)	36	3	9	8.3 (2-23)	25.0 (14-41)
LM (L'hoest monkey)	8	6	8	75.0 (40-94)	100 (63-100)
OB (Olive baboon)	27	18	27	66.7 (48-81)	100 (85-100)
RC (Red colobus)	64	11	26	17.2 (10-28)	40.6 (29-53)
RT (Red-tailed guenon)	41	21	38	51.2 (36-66)	92.7 (80-98)
TOTAL	318	112	222	35.2 (30-40)	69.8 (65-75)

Table 2.2: Univariate PGLS models. Phylogenetic generalized least-squared multiple regression models of the relationship between *Oesophagostomum* prevalence and univariate life-history variables of diurnal primates (excluding humans) within the Kibale community

Univariate Model	λ	Slope	F	*p*	Adjusted r^2
Terrestriality	0.97	0.20495	2.93	0.130	0.2159
Home range	1.00	0.00004	1.07	0.400	0.0101
Group size	1.00	-0.00023	0.05	0.950	-0.1567
Polyspecific association	1.00	-0.19713	1.16	0.375	0.0225
Body mass	1.00	0.00400	0.61	0.578	-0.0590
Log daily travel	0.00	0.18500	9.43	0.014	0.5464

Table 2.3: Multivariate PGLS models. Phylogenetic generalized least-squared multiple regression models of the relationship between *Oesophagostomum* prevalence and life-history variables of diurnal primates (excluding humans) within the Kibale community

Multivariate Model	λ	Slope	t	*p* trait	F	*p* overall	Adjusted r^2
y-intercept	0.00	-1.93390	-2.65	0.045	9.95	0.015	0.7189
Home range +		0.00012	-2.16	0.083			
Log daily travel		0.00039	3.70	0.014			
y-intercept	0.00	-0.89378	-2.58	0.049	12.73	0.009	0.7701
Group size +		-0.00180	-2.61	0.047			
Log daily travel		0.24894	5.04	0.004			
y-intercept	0.00	-1.48140	-1.97	0.120	8.39	0.032	0.7599
Home range +		-0.00006	-0.88	0.425			
Group size +		-0.00127	-1.36	0.245			
Log daily travel		0.33085	3.14	0.035			

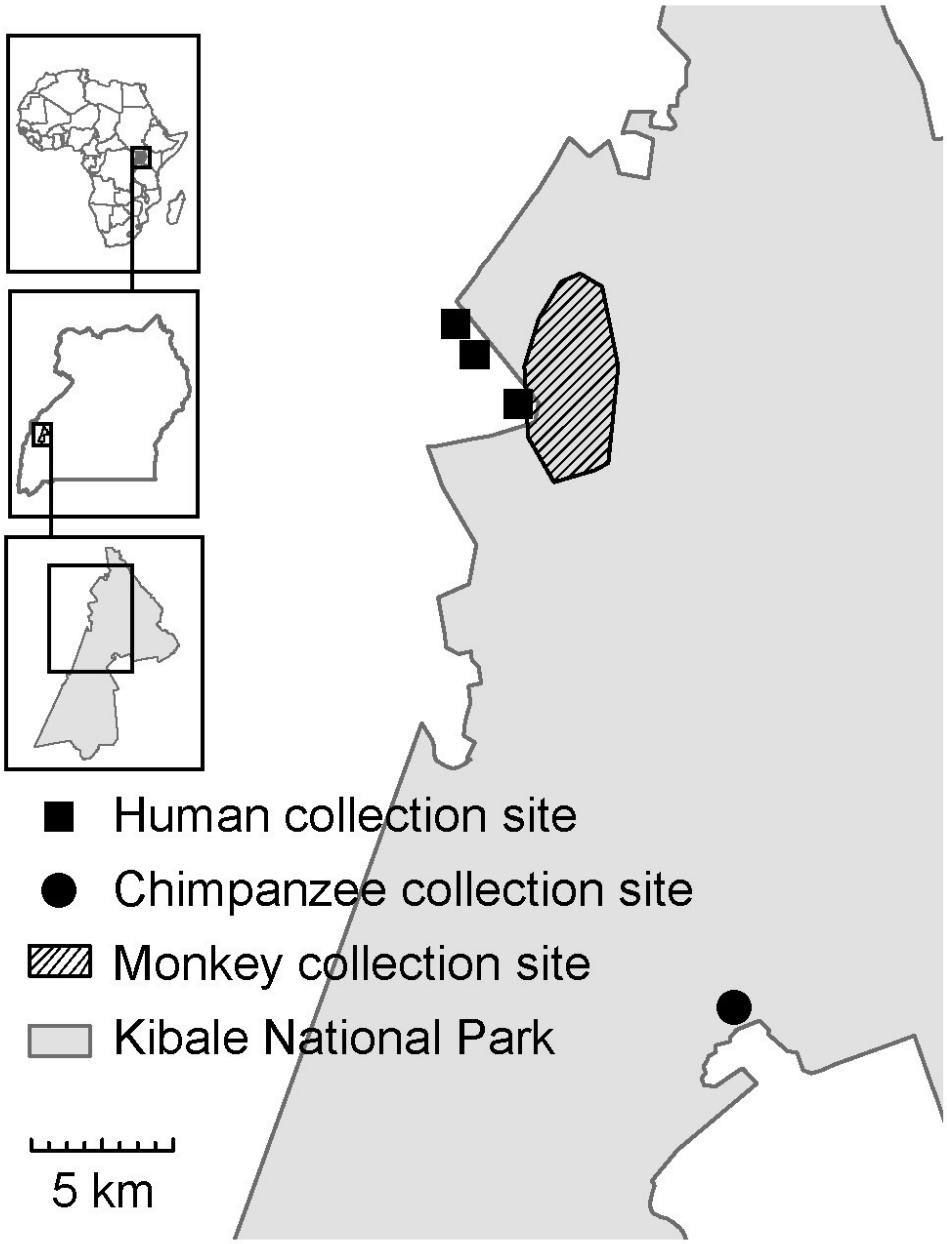

Figure 2.1: **Sample collection sites in and around Kibale National Park, Uganda.** Wild primates that were collected from included: chimpanzees (*Pan troglodytes*), baboons (*Papio anubis*), black-and-white colobus (*Colobus guereza*), blue monkeys (*Cercopithecus mitis*), grey-cheeked mangabeys (*Lophocebus albigena*), l'hoest monkeys (*Cercopithecus lhoesti*), red colobus (*Procolobus rufomitratus*) and red-tailed guenons (*Cercopithecus ascanius*)

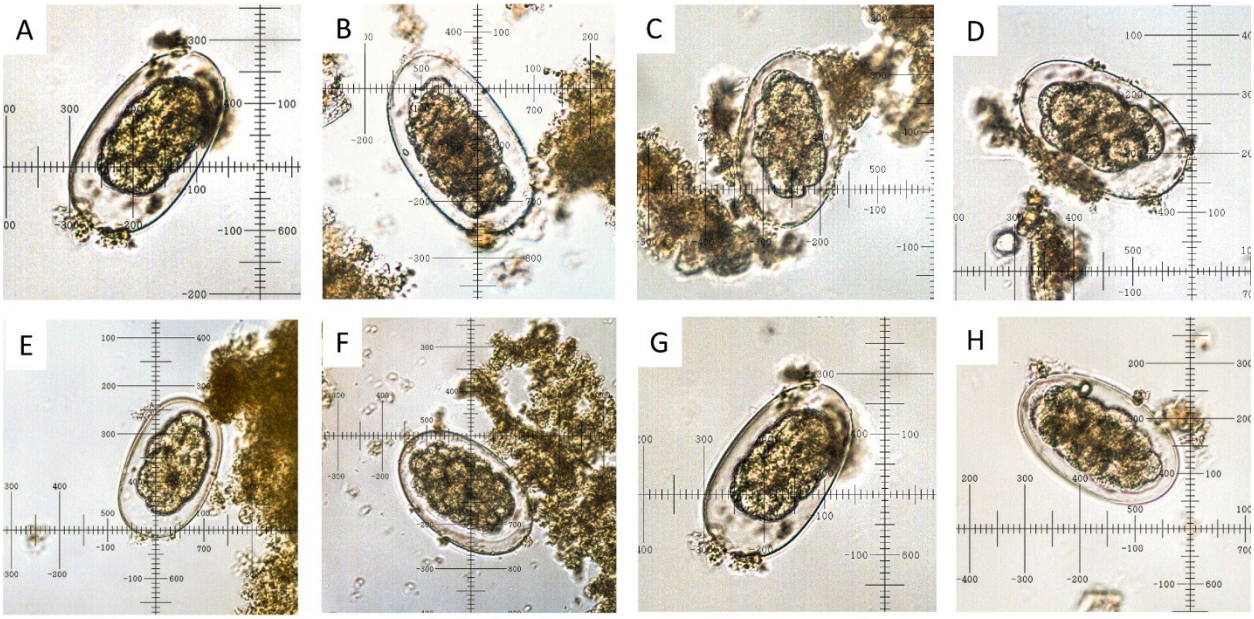

Figure 2.2: Representative *Oesophagostomum* eggs from eight hosts. Microscopic images of representative *Oesophagostomum* sp. eggs found in the feces of infected primate hosts. Images were captured at 40X objective magnification from thin smears of sedimented feces. A = blue monkey, B = black-and-white colobus, C = chimpanzee, D = l'hoest monkey, E = grey-cheeked mangabey, F = olive baboon, G = red-tailed guenon, H = human. All eggs were between 65 and 84 µm long and between 35 and 55 µm wide.

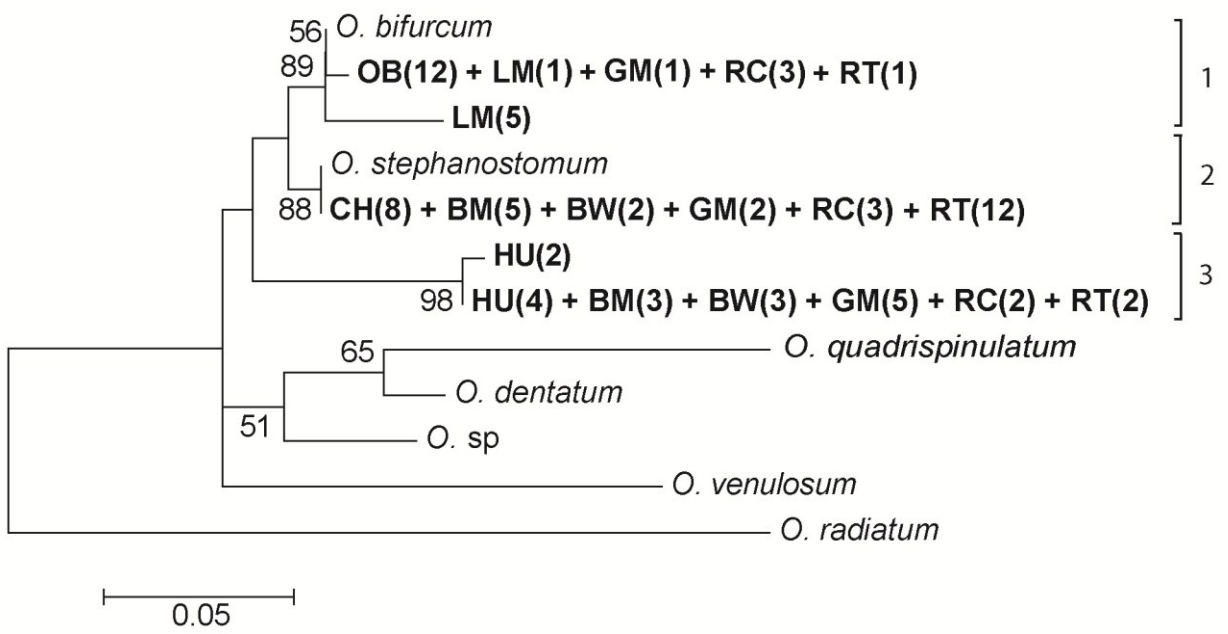

Figure 2.3: Phylogenetic tree of *Oesophagostomum*. Phylogenetic analysis of *Oesophagostomum* based on ITS2 rDNA (260 bp) sequences. Nucleotide sequences were aligned using Clustal X software (Larkin *et al.* 2007). Phylogenetic relationships were inferred in MEGA5 (Tamura *et al.* 2011), using the maximum likelihood method with a Hasegawa-Kishino-Yano model of nucleotide substitution (Hasegawa *et al.* 1985). The best-scoring maximum-likelihood tree is shown here (-lnL = 656.5). Bootstrap values (%) greater than 50% are shown. Taxon names of sequences generated in this study are in bold and correspond to the host species followed by the number of infected individuals in parentheses (BM = blue monkey, BW = black-and-white colobus, CH = chimpanzee, GM = grey-cheeked mangabey, HU = human, LM = l'hoest monkey, OB = olive baboon, RC = red colobus, and RT = red-tailed guenon). Reference sequences correspond to Genbank accession numbers AF136575 and Y11733 for *O. bifurcum*, AF136576 for *O. stephanostomum*, HQ844232 for *O. sp*, Y11738 for *O. quadrispinulatum*, Y11735 for *O. dentatum*, Y10790 for *O. venulosum*, and AJ006149 for *O. radiatum*. Scale bar indicates nucleotide substitutions per site.

LINKING STATEMENT 1

In the previous chapter, I examined the host range of a soil transmitted helminth, *Oesophagostomum*, within the primate community and adjacent human population. I discovered three lineages of *Oesophagostomum*. Two lineages matched to previously characterized species – *O. bifurcum* and *O. stephanostomum*. The third lineage, however, was genetically uncharacterized but appeared to be transmissible between humans and a number of monkey species. These findings are contrary to predictions based on existing theory. Soil-transmitted helminths are physiologically complex and have slow generation times that may prevent adaptation to new hosts, so I predicted that *Oesophagostomum* would be a specialist parasite.

I also predicted that *Oesophagostomum* would infect hosts based on their phylogenetic relatedness to one another, such that closely related hosts would share similar or the same parasites. One lineage agreed with this prediction, as it was only found in monkeys, and not apes (this lineage was phylogenetically clustered in NRI analysis). The other two lineages did not show patterns of phylogenetically structured transmission, however. Instead, overall prevalence of infection was more related to the life history traits of primates, specifically daily travel distance and group size. These results suggest that factors that put primates at risk of exposure to infective-stage parasites are more important than host relatedness for transmission of this parasite.

In the next chapter, I characterize the host range and transmission of another soil transmitted helminth, *Trichuris*. *Oesophagostomum* and *Trichuris* are similar in both mode of transmission and life history. Therefore, I expect similar patterns of transmission between these parasites. I used the same samples in the next chapter as those collected in the previous. In doing so, I can directly compare the transmission of these two similar parasites while controlling for differences in sampling within the community (Appendix A).

CHAPTER 3

Hidden population structure and cross-species transmission of whipworms (*Trichuris* sp.) in humans and non-human primates in Uganda

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3.1 Abstract

3.1.1 Background

Whipworms (*Trichuris* sp.) are a globally distributed genus of parasitic helminths that infect a diversity of mammalian hosts. Molecular methods have successfully resolved porcine whipworm, *Trichuris suis*, from primate whipworm, *T. trichiura*. However, it remains unclear whether *T. trichiura* is a multi-host parasite capable of infecting a wide taxonomic breadth of primate hosts, or a complex of host-specific parasites that infect one or two closely related hosts.

3.1.2 Methods and Findings

We examined the phylogenetic structure of whipworms in a multi-species community of non-human primates and humans in western Uganda, using both traditional microscopy and molecular methods. A newly developed nested polymerase chain reaction (PCR) method applied to non-invasively collected fecal samples detected *Trichuris* with 100% sensitivity and 97% specificity relative to microscopy. Infection rates varied significantly among host species, from 13.3% in chimpanzees (*Pan troglodytes*) to 88.9% in olive baboons (*Papio anubis*). Phylogenetic analyses based on nucleotide sequences of the *Trichuris* internal transcribed spacer regions 1 and 2 of ribosomal DNA revealed three co-circulating *Trichuris* groups. Notably, one group was detected only in humans, while another infected all screened host species, indicating that whipworms from this group are transmitted among wild primates and humans.

3.1.3 Conclusions

Our results suggest that the host range of *Trichuris* varies by taxonomic group, with some groups showing host specificity, and others showing host generality. In particular, one *Trichuris* taxon should be considered a multi-host pathogen that is capable of infecting wild primates and humans. This challenges past assumptions about the host specificity of this and similar helminth parasites and raises concerns about animal and human health.

3.2 Introduction

Parasites that infect multiple host species are of particular concern because they are more likely to emerge than single-host parasites (Gao *et al.* 1999; Taylor *et al.* 2001; Woolhouse *et al.* 2001; Leroy *et al.* 2009). Moreover, multi-host parasites are difficult to control because reservoir hosts may serve as sources of re-infection for other populations in which the parasite has been eliminated (Anderson & May 1992; Dupouy-Camet 2000; Gottstein *et al.* 2009). A number of ecological and evolutionary factors influence the range of hosts that a parasite can infect (host specificity). Multi-host parasites of non-human primates (hereafter primates) have come under particular scrutiny, because physiological similarity (due to relatedness) between primates and humans increases the potential for zoonotic transmission. Indeed, phylogenetic relatedness between primate hosts is a stronger predictor of parasite sharing than geographic overlap (Davies & Pedersen 2008). Despite the probability of parasite sharing between primates and humans, only 20% of primate helminths (parasitic worms) are thought to infect humans (Pedersen *et al.* 2005). Conversely, half of all primate helminths are thought to be specific to a single host species (Pedersen *et al.* 2005; Nunn & Altizer 2006). These observations suggest that, compared to other taxonomic groups of parasites, helminths have a lesser propensity for zoonotic transmission, perhaps because of their physical complexity, indirect life cycles, and long generation times (Anderson & May 1992; Cleaveland *et al.* 2001).

Here we examine the host specificity of the whipworm genus *Trichuris*, a soil-transmitted helminth with a global distribution (Bethony *et al.* 2006). *Trichuris trichiura* is estimated to affect approximately 600 million people worldwide (Hotez *et al.* 2007; Hotez *et al.* 2009), causing physical and mental growth retardation in children (Stephenson *et al.* 2000; Bethony *et al.* 2006). *Trichuris* infection results from ingestion of embryonated eggs shed into food, water, and soil (Stephenson *et al.* 2000). Following ingestion, first-stage larva (L1s) hatch and move through the gastrointestinal tract where they develop in the caecum, molt into adults, and tunnel into the mucosa of the large intestine. After mating, female whipworms release eggs into feces. Eggs typically become infective after 20 days or more in the environment, where they are tolerant to desiccation and temperature extremes (Nolf 1932; Burden & Hammet 1976; Gilman *et al.* 1983; Holland 1987; Bundy & Cooper 1989).

Currently, the *Trichuris* genus contains more than 20 described species that are generally specific to taxonomic groups of hosts (Bundy & Cooper 1989). Traditional parasitological research on the genus has focused on differentiating *Trichuris trichiura*, found in humans and primates, from *Trichuris suis*, found in pigs (Beer 1976; Ooi *et al.* 1993; Cutillas *et al.* 2009; Liu *et al.* 2012a; Nissen *et al.* 2012). Morphologically, these two species are similar, and previous attempts to distinguish them based on variation in reproductive organ morphology were inconclusive because phenotypic plasticity could not be distinguished from genotypic differences (Oliveros *et al.* 2000). The unsuitability of morphological characteristics for resolving differences between *T. trichiura* and *T. suis* made molecular methods a promising approach. Sequences from the internal transcribed spacer regions 1 and 2 (ribosomal DNA) from primate and porcine hosts suggest that *T. trichiura* and *T. suis* are two closely related but separate species (Cutillas *et al.* 2009), a conclusion further supported by subsequent analyses of β tubulin gene sequences (Nissen *et al.* 2012).

Morphological studies of *Trichuris* isolated from primates and humans conclude that the species infecting these hosts is the same, despite slight morphological variations that are distinguishable using scanning electron microscopy (Ooi *et al.* 1993). These results suggest that both primates and humans are infected with *T. trichiura*, which is capable of freely switching between primate and human hosts. Perhaps as a result of these findings, DNA sequences isolated from both primate and human hosts have been assumed to be *T. trichiura* by virtue of the host alone, and without the taxonomic scrutiny required to identify the parasite to species level. An empirical test of the assumption that primate and human *Trichuris* are identical used molecular methods to sequence DNA from *Trichuris* adults isolated from chacma baboons (*Papio ursinus*) and humans. Results revealed two distinct lineages of *Trichuris* in baboons (Ravasi *et al.* 2012). The authors concluded that both lineages were transmissible between humans and baboons, and that *T. trichiura*, while perhaps not a single lineage, is a zoonotic parasite. Transmission between humans and primates is additionally supported by a molecular study of both β tubulin and ITS 2 gene regions isolated from both humans and baboons (*Papio anubis, P. hamadryas*), where no genetic differentiation between host species was found (Hansen *et al.* 2013). In contrast, work on both ribosomal DNA and complete mitochondrial genome sequences has found evidence of host specificity within the *Trichuris trichiura* species complex (Liu *et al.* 2013). These results led to

the suggestion that *Trichuris trichiura* is not a single multi-host parasite, but rather a complex of host-specific lineages, each infecting distinct taxonomic groups of primates (Liu *et al.* 2013). This suggestion is supported by molecular data from a small number of studies in non-human primate taxa (Cutillas *et al.* 2002; Cutillas *et al.* 2007; Liu *et al.* 2012a; Liu *et al.* 2012b; Nissen *et al.* 2012; Liu *et al.* 2013).

In this study, we examine the phylogenetic structure of *Trichuris* in a host community comprised of wild primates and a nearby human population. Our study is based in and around Kibale National Park, Uganda, where *Trichuris* is known to infect several species (Gillespie *et al.* 2004, 2005b; Chapman *et al.* 2006; Bezjian *et al.* 2008; Goldberg *et al.* 2008b). Humans and primates in this region frequently overlap. For example, several species of primates raid crops, and people often enter the park to extract resources such as wood, food, and traditional medicines (Naughton-Treves 1997; Naughton 1998; Naughton-Treves & Chapman 2002; Hartter 2010). People and primates are exposed to the same physical environment during such events and can even interact directly (Goldberg *et al.* 2012). Thus, the Kibale ecosystem is useful for examining the host specificity of parasites in a setting where cross-species transmission, including zoonotic transmission, is ecologically possible. Indeed, previous research in Kibale has demonstrated cryptic genetic lineages and cross-species transmission of another soil-transmitted helminth genus of primates and humans, the nodule worm (*Oesophagostomum* spp.) (Ghai *et al.* 2014). Our results herein demonstrate that the taxonomy and population structure of *Trichuris* is more complex than previously appreciated. Specifically, we identify cryptic *Trichuris* lineages, of which some infect multiple primate host species, including humans.

3.3 Methods

3.3.1 Study Site and Sample Collection

Kibale National Park (0°13'-0°41'N, 30°19'-30°32' E) is a 795 km^2 mid-altitude rainforest located in Western Uganda. Kibale harbors nine species of diurnal primate that have been the focus of over four decades of research on primate ecology (Struhsaker 1997; Chapman & Lambert 2000; Chapman *et al.* 2005b; Chapman *et al.* 2010), and infection, including zoonoses (Goldberg *et al.* 2008c; Johnston *et al.* 2010; Yildirim *et al.* 2010; Lauck *et al.* 2011; Goldberg *et al.* 2012; Lauck *et al.* 2012; Salyer *et al.* 2012; Thurber *et al.* 2013). Kibale is surrounded by a

dense human population of up to 600 people/km^2 (Mackenzie & Hartter 2013). Sample collection occurred in and around Kanyawara, a North Western segment of the park (see Ghai *et al.* (2014)).

Trichuris and other gastrointestinal helminths pass their eggs in the feces of their host, which offers an opportunity to conduct molecular analysis non-invasively by isolating DNA directly from parasite eggs. We collected primate fecal samples non-invasively from individuals in habituated primate groups. All primate groups were sampled only once to prevent pseudo-replication of individuals. Fecal samples were collected immediately after defecation and placed in sterile tubes. Seven diurnal monkey species were sampled: black-and-white colobus (*Colobus guereza*), blue monkeys (*Cercopithecus mitis*), grey-cheeked mangabeys (*Lophocebus albigena*), l'hoest monkeys (*Cercopithecus lhoesti*), olive baboons (*Papio anubis*), red colobus (*Procolobus rufomitratus*), and red-tailed guenons (*Cercopithecus ascanius*). Chimpanzee (*Pan troglodytes*) samples were collected from two habituated groups in Kanyanchu, a section of Kibale approximately 15 km from Kanyawara. Human samples were collected from individuals between the ages of 2 and 70 residing in one of three villages within 5 km of the park boundary. Following informed consent, participants were provided with collection materials and instructions, and samples were retrieved within one day for processing.

All samples underwent a procedure to concentrate nematode eggs while removing particles and debris. A modified ethyl acetate sedimentation method using one gram of feces was chosen due to its suitability for field conditions and its efficacy at recovering helminths eggs (Garcia *et al.* 2005). Details are provided elsewhere (Ghai *et al.* 2014). Samples were collected between May and August 2011.

3.3.2 Microscopy

We used microscopy to confirm infection status by identifying *Trichuris* eggs. Thin smears of sedimented feces were examined under 10X objective magnification on a Leica DM2500 light microscope. Length, width, color, and contents of eggs were recorded at 40X magnification, and images were captured with an Infinity1 CMOS digital microscope camera and Infinity Camera v.6.2.0 software (Lumenera Corporation, Ottawa, ON, Canada). Samples were considered

positive for *Trichuris* when one or more eggs with the characteristic *Trichuris* "lemon" shape were identified. Samples were considered free of *Trichuris* only after the entire sediment was scanned and no *Trichuris* eggs were seen. All samples were examined by the same observer (RRG) to avoid inter-observer bias.

3.3.3 Molecular Methods

DNA was extracted from 200 µl of sedimented feces preserved in RNAlater nucleotide stabilization solution (Sigma-Aldrich, St. Louis, MO, USA) using a ZR Fecal DNA MiniPrep Kit (Zymo Research Corporation, Irvine, CA, USA), following manufacturer protocols.

The parasite internal transcribed spacer region (ITS) 1 of the ribosomal DNA complex was amplified using polymerase chain reaction (PCR) with newly designed primers that were specific to the genus *Trichuris*. These primers were nested within the 18S (small ribosomal subunit) coding region and the 5.8S non-coding region (see Romstad *et al.* (1997)). Two forward primers (external and internal) were sited within conserved regions of 18S sequences of *T. trichiura* (Genbank accession numbers: AB699091, AB699090, AB699092, GQ352548), *T. suis* (accession no. AY851265), *T. vulpis* (accession no. GQ352558), and *T. muris* (accession no. AF036637). Other enoplean nematodes (*Romanomermis*, accession no. AY146544; *Agamermis*, accession no. DQ628908; *Capillaria*, accession no. EU004822; and *Trichinella*, accession no's. U60231 and AY487254), as well as representative genera likely to occur in Kibale (*Caenorhabditis*, accession no. JN636068; *Strongyloides*, accession no. M84229) were included in primer design alignments to ensure primers were specific to *Trichuris*. The two generated primers were: External_*Trichuris*-1417F (5'-AGGGACCAGCGACACTTTC-3') and Internal_*Trichuris*-1567F (5'-GTTCTCGTGACTGGGAC-3').

Reverse primers that were also specific for the genus *Trichuris* were designed in a similar manner, using aligned 5.8S sequences from *T. trichiura* (accession no's. GQ301555, GQ301554, KC877992), *T. suis* (accession no's. JF690951, AM993015), *T.* sp (accession no's. JF690940-JF690952, HQ844233), *T. ovis* (accession no. JX218218), *T. muris* (accession no. FN543201), *T. arvicolae* (accession no. FR849687), and *T. discolor* (accession no. JX281223). Other enoplean nematodes (*Trichinella*, accession no's. AF342803, KC006431) and representative genera likely

to be found in Kibale (*Oesophagostomum*, accession no's. AJ619979 and AB821014; *Strongyloides*, accession no. EF653265; *Xiphinema*, accession no. HM990158) were also included. The reverse primers generated were: ExternalITS1_*Trichuris*-2505R (5'-GAGTGTC ACGTCGTTCTTCAAC-3') and InternalITS1_*Trichuris*-2462R (5'-CTACGAGCCAAG TGATCC-3'). External primers generated amplicons of approximately 1088 bp expected size; internal primers generated amplicons of 895 bp expected size.

The ITS 2 region was amplified using primers nested within the 5.8S non-coding and 28S (large ribosomal subunit) coding regions. The ITS 1 internal reverse primer described above (InternalITS1_*Trichuris*-2462R) was reversed and used as the forward external primer (ExternalITS2_*Trichuris*-2462F: 5'-GGATCACTTGGCTCGTAG-3'). The internal primer, InternalITS2_*Trichuris*-2560F (5'-CTTGAATACTTTGAACGCACATTG-3') was designed using the aligned 5.8S sequences described above and was also specific to the genus *Trichuris*. A previously published, conserved primer NC2 (5'-TTAGTTTCTTTTCCTCCGCT-3') was used as the reverse primer in both external and internal reactions (Gasser *et al.* 1993). External primers generated amplicons of approximately 584 bp expected size; internal primers generated amplicons of 486 bp expected size.

The efficacy of the protocols designed for amplifying only *Trichuris* ITS 1 and 2 regions was tested using dilutions of a positive control (adult *T. vulpis* isolated by necropsy from an infected canine at Cornell University), and by implementing the protocol on samples known to contain infections with multiple parasite genera. The protocol was found to be 100% accurate at detecting only *Trichuris* even among mixed infections.

ITS 1 external PCR was performed in 25 µL volumes using the FailSafe System (Epicentre Biotenchnologies, Madison, WI, USA). Reactions contained 1X FailSafe PCR PreMix with Buffer C (containing dNTPs and $MgCl_2$), 1 Unit of FailSafe Enzyme Mix, 2.5 picomoles of each primer (ExternalITS1_*Trichuris*-1417F and ExternalITS1_*Trichuris*-2505R), and 1 µL of template (extracted DNA from sedimented feces). Reactions were cycled in a Bio-Rad CFX96 thermocycler (Bio-Rad Laboratories, Hercules, CA, USA) with the following temperature profile: 94°C for 60 sec; 40 cycles of 94°C for 60 sec, 61°C for 30 sec, 72°C for 75 sec; and a

final extension at 72°C for 10 min. Internal PCR was performed in 25 µL volumes using the DyNAzyme DNA Polymerase Kit (Thermo Scientific, Asheville, NC, USA) with reactions containing 0.5 Units of DyNAzyme I DNA Polymerase, 1X Buffer containing 1.5 mM $MgCl_2$, 2.5 picomoles of each primer (InternalITS1_*Trichuris*-1567F and InternalITS1_*Trichuris*-2462R)), 0.5 µL dNTPs, and 1 µL of template (product of the external PCR reaction). Reactions were cycled according to the following temperature profile: 94°C for 60 sec; 35 cycles of 94°C for 30 sec, 55°C for 30 sec, 72°C for 75 sec; and a final extension at 72°C for 10 min.

ITS 2 PCR used the same reagents as the ITS 1 external and internal reactions described above, with external reactions using ExternalITS2_*Trichuris*-2462F and NC2 primers, and internal reactions using InternalITS2_*Trichuris*-2560F and NC2 primers. Both external and internal reactions were cycled according to the following temperature profile: 94°C for 60 sec; 35 cycles of 94°C for 30 sec, 55°C for 30 sec, 72°C for 60 sec; and a final extension at 72°C for 10 min. PCR products were electrophoresed on 1% agarose gels stained with ethidium bromide. Amplicons were excised and purified using the Zymoclean Gel DNA Recovery Kit (Zymo Research Corporation, Irvine, CA, USA) according to the manufacturer's instructions.

ITS 1 and 2 products were Sanger sequenced in both directions using primers InternalITS1_*Trichuris*-1567F and InternalITS1_*Trichuris*-2462R for ITS 1 and InternalITS2_*Trichuris*-2560F and NC2 for ITS 2. Sequencing was performed on ABI 3730xl DNA Analyzers (Applied Biosystems, Grand Island, NY, USA) at the University of Wisconsin-Madison Biotechnology Center DNA Sequencing Facility. Sequences were hand-edited and assembled using Sequencher v. 4.9 (Gene Codes Corporation, Ann Arbor, MI, USA) with reference to published sequences. Generation of unambiguous sequences required repeat PCR and re-sequencing on three occasions. Newly generated sequences were deposited in GenBank, under accession numbers KJ588071-KJ588132 (18S, ITS 1) and KJ588133-KJ588167 (5.8S, ITS 2, 28S).

3.3.4 Analyses

The ratio of *Trichuris* egg length to width was calculated and compared among groups using Kruskal-Wallis tests and Dunn's multiple comparison post-tests in Prism6 (GraphPad Software

Inc., La Jolia, CA, USA) to assess shape differences between different groups of *Trichuris*. To compare the diagnostic performance of microscopy with newly designed PCR methods, sensitivity (*i.e.*, the proportion of samples correctly identified as positive by PCR as compared to microscopy) and specificity (*i.e.*, the proportion of samples correctly identified as negative by PCR) were calculated using MedCalc v. 12.5.0 (MedCalc Software, Ostend, Belgium). Prevalence of *Trichuris* infection was calculated as the total number of positive samples divided by the total number of samples, with 95% confidence intervals calculated using the modified Wald method (Agresti & Coull 1998). Differences in prevalence among host species were evaluated using Fisher's exact tests implemented in the program Quantitative Parasitology v. 3.0 (Rozsa *et al.* 2000).

Due to the number and varying sizes of indels among DNA sequences, we aligned sequences using webPRANK, a phylogeny-aware progressive alignment tool that has been shown to outperform other methods in indel-rich alignment (Löytynoja & Goldman 2008, 2010). Aligned sequences were trimmed to consistent length and missing data were coded as "?" in BioEdit v. 7.2.5 (Hall 1999). Samples for which both ITS 1 and 2 were generated were concatenated in Sequence Matrix v. 1.7.8 (Vaidya *et al.* 2011). All sequences were subjected to Gblocks treatment to remove regions of ambiguous alignment using the following parameters: "Maximum number of contiguous non-conserved positions" = 100, "Minimum length of a block" = 4, and "Allowed gap positions" = half (Castresana 2000). Models of sequence evolution for each gene were selected using the MrModelTest v. 2 executable in PAUP* v. 4 (Swofford 2003; Nylander 2004).

We reconstructed phylogenetic relationships using Bayesian methods and HKY + I (ITS 1) and HKY (ITS 2) models, implemented in MrBayes v. 3.2.2 through the CIPRES Science Gateway (Ronquist & Huelsenbeck 2003; Miller *et al.* 2010). Phylogenetic analyses were conducted on concatenated, Gblocks treated ITS 1 and 2 sequences. Four chains were run for 1 X 10^7 MCMC generations, sampling every 1000^{th} generation with a diagnostic frequency of every 5000^{th} generation. MCMC runs continued until a standard deviation of split frequency value of 0.01 was reached. Convergence was confirmed when all substitution model parameters reached a potential scale reduction factor value of 1, and was visually assessed using Tracer v. 1.6. The first 10% of

runs were discarded as burn-in and Bayesian posterior probabilities were calculated from the remaining trees.

Genetic divergence among *Trichuris* populations was estimated as percent nucleotide-level sequence identity, calculated as the uncorrected pairwise proportion of nucleotides (*p*-distance) in MEGA v. 5.1 with 1000 bootstrap replicates (Tamura *et al.* 2011). Analysis of molecular variance (AMOVA) was used to partition *Trichuris* genetic diversity into within host and between host components (Excoffier *et al.* 1992) in GenAlEx v. 6.5 (Peakall & Smouse 2012). Pairwise population differentiation values (PhiPT; an analogue of FST), were also calculated in GenAlEx. To assess the relationship between host phylogeny and parasite phylogeny, mantel tests were used to compare pairwise distance matrices of phylogenetic branch lengths between primate hosts and *p*-distance among parasite clades (calculated as described above) using the ape package (Paradis *et al.* 2004) in the statistical programming language R (Development-Team 2008).

3.4 Results

We collected 282 samples from primates and 36 samples from humans, for a total of 318 samples. Of these, microscopy classified 104 samples as *Trichuris*-positive, making the community-wide prevalence of infection by microscopy 32.7% (Table 1). Eggs varied considerably in length (50-76μm), and width (26-30μm), but length-to-width ratios did not differ significantly among parasite clades (see below) or host species (Kruskall-Wallis test, $P > 0.05$; Figure 3.1).

PCR of ITS 1 and ITS 2 generated single, clear bands of expected size. PCR of ITS 1 and 2 generated identical results and were therefore considered together for the purposes of evaluating the diagnostic performance of PCR. PCR correctly classified all samples that were positive for *Trichuris* by microscopy. In addition, PCR classified five samples as positive for *Trichuris* that were negative by microscopy. Thus, the sensitivity of our new PCR assay was 100% (95% C.I. 96.5%-100.0%) and the specificity was 97.7% (95% C.I. 94.6%-99.2%), suggesting that our new PCR assays of ITS 1 and 2 are both highly accurate.

Prevalence varied significantly by species, (χ^2 = 62.99, df = 8, P < 0.0001), with chimpanzees (13.3%) and grey-cheeked mangabeys (14.3%) having the lowest prevalence, and olive baboons (88.9%) the highest (Table 1).

Of the 108 positive samples, 74 samples were selected for sequencing to represent the widest possible range of host species and, to the greatest extent possible, to equalize sequencing effort among host species. Because preliminary results indicated that ITS 1 provided greater phylogenetic resolution than ITS 2, 62 sequences for ITS 1 and 35 sequences for ITS 2 were ultimately generated. In samples where both ITS 1 and ITS 2 sequences were generated, sequences were concatenated and gaps were coded as missing data. The final alignment length of Gblock treated and concatenated ITS 1 and ITS 2 sequences was 1083 characters.

Phylogenetic analysis resolved *Trichuris* into three groups, which, for convenience, we designate Groups 1, 2 and 3 in Figure 3.2. Group 1 contained two samples from humans that were 98.2% identical to each other and that most closely matched published sequences from Chacma baboons (*Papio hamadryas ursinus*) from South Africa (Genbank accession numbers GQ301551-2 (Ravasi *et al.* 2012). This clade, along with a sequences from humans in Uganda (Nissen *et al.* 2012) (Genbank accession numbers JN181837, JN181845), are sister to the *Trichuris* in-group and are the most genetically divergent lineage, sharing between 71.7% and 88.1% nucleotide similarity with other *T. trichiura* clades (Figure 3.2). Group 2 contained sequences from four black-and-white colobus and one red colobus that shared 100% nucleotide identity, and were most closely related to published *Trichuris* sequences from another subspecies of black-and-white colobus (*Colobus guereza kikuyuensis*) and yellow-cheeked gibbons (*Nomascus gabriellae*) from a zoo in Spain (Cutillas *et al.* 2009). Finally, Group 3 contained 59 sequences from all seven species of primate host and eight humans sampled in this study. This group shared 99.3% nucleotide sequence identity and clustered most closely with published sequences from humans in Cameroon (accession number GQ301555), and more distantly with Chacma baboons in South Africa from the same study (accession number (GQ301554) (Ravasi *et al.* 2012). All three sequences representing *T. suis* clustered within the *T. trichiura* species complex, and were most distinct (excluding outgroups) from Group 1 (66.9% nucleotide similarity), and most similar to Group 2 (88.5% nucleotide similarity).

Samples from human hosts identified in this study fell within Groups 1 and 3. Human-derived *Trichuris* sequences were most similar to those from grey-cheeked mangabey (95.2% similarity) and chimpanzees (95.1% identity), and most dissimilar to those of black-and-white colobus (91.2% identity; Table 2). When within-group sequence variation was held constant in PhiPT analysis, sequences from black-and-white colobus and olive baboons were significantly different, but sequences from other species pairs were not (Table 2). Mantel tests comparing host phylogeny and parasite *p*-distances between clades were not significant (Z-statistic = 43.37, p = 0.305). AMOVA revealed that 98% of *Trichuris* sequence-level variation was contained within host species, with only 2% of sequence-level variation apportioned between host species.

3.5 Discussion

We investigated the taxonomy and phylogenetic structure of the whipworm genus *Trichuris* in a wild primate community and a nearby human population in Uganda. The overall prevalence of infection was 34%, but this varied significantly among host species, with the lowest prevalence in chimpanzees (13.3%) and the highest prevalence in olive baboons (88.9%). Research in Gombe National Park, Tanzania, where these two species also overlap, found similar results, with chimpanzees having 5% infection prevalence and baboons 66% (Murray *et al.* 2000). Averaging across sites in Tanzania and Senegal, another study found prevalences of 4.5% and 35% in chimpanzees and baboons, respectively. Interestingly, *Trichuris* is one of the few parasites with consistently higher prevalence in baboons than in chimpanzees (McGrew *et al.* 1989). In an attempt to explain interspecific differences in prevalence, we conducted a phylogenetic-least-squared regression to explore correlations between host traits (terrestriality, home range, group size, time spent in polyspecific associations, body mass, and daily travel distance) and prevalence (not shown), but found no significant relationships. It therefore remains unclear why *Trichuris* prevalence varies significantly among sympatric primate hosts.

In humans, our results indicate a prevalence of 30.6% by PCR. Previous research in Uganda has estimated prevalence to be between 12.9 and 28% using microscopy; however, this is among school-aged children, where the frequency of *Trichuris* infection is high (Kabatereine *et al.* 1997; Standley *et al.* 2009). Our estimate of 30.6% infection in a human community containing

individuals of multiple ages suggests that this region of Uganda has a generally high rate of infection. Poor access to latrines, earthen flooring in houses, and large family sizes are likely contributing factors (Narain *et al.* 2000), although improved accuracy of our methods relative to others may also help explain this difference.

Our phylogenetic analysis revealed that *Trichuris* sequences from the Kibale primate community and neighboring human population sorted into three groups. Group 1 contained two sequences from humans and clustered closely with sequences derived from Chacma baboons (Ravasi *et al.* 2012). Interestingly, these sequences were designated as part of the most phylogenetically distinct *T. trichiura* clade, most distant from *T. suis* [27]. The authors of these sequences therefore refrained from designating this clade *T. trichiura* (Ravasi *et al.* 2012). Our results support the conclusion that this *Trichuris* clade represents a separate species, since our phylogenetic analysis placed Group 1 and associated published reference sequences as sister to all other *Trichuris* in-groups. Our *p*-distance analyses similarly estimate the maximum dissimilarity between Group 1 (and associated published reference sequences) and all other in-groups to be 33.1%, which is nearly twice that between previously described sequences of *T. trichiura* and *T. suis*, which are recognised as taxonomically distinct species.

Group 1 sequences and GQ301551-2 were sister to sequences that were part of study that sought to identify genetic similarity between *T. trichiura* and *T. suis* derived from humans and pigs living in close proximity (Nissen *et al.* 2012). In the latter study, two distinct genotypes of human-derived *Trichuris* were defined, which they designated type 1 and type 2 (Nissen *et al.* 2012). Our sequences cluster with their type 1 genotype (represented by JN181860 in Figure 3.2), the clade more distantly related to *T. suis*. Despite screening the entire diurnal primate community, we detected Group 1 only in humans. However, a similar genotype has been found elsewhere in baboons [27], suggesting that the Group 1 lineage may have a broader host range than documented in our study, perhaps indicating a potential for infrequent cross-species transmission.

In contrast to *Trichuris* Group 1, we detected *Trichuris* Group 3 in every host species sampled, including humans. This result suggests that Group 3, including published reference sequences,

represents a multi-host lineage capable of infecting multiple primate hosts, including humans. Our population analyses support these results, in that only 2% of overall *Trichuris* genetic variation is apportioned between host species.

Group 2, containing sequences derived from black-and-white colobus and red colobus, also appears to be a distinct lineage. This clade is most closely related to *T. trichiura* from other primates, namely gibbons and another subspecies of black-and-white colobus (Cutillas *et al.* 2009). This *Trichuris* lineage may have an intermediate host range compared to Groups 1 and 3, given that all samples save one were derived from colobus monkeys. The one sample that was not derived from colobines (gibbon) was collected from a zoo, and may therefore reflect transmission outside of a natural setting. Additional sampling and sequencing would help clarify the host range of this *Trichuris* taxon.

We note that rDNA occurs in multiple copies, and this study does not attempt to quantify intraspecific variation or mixed lineage infections. Our data therefore reflect a minimum conservative estimate of parasite genetic variation. Similarly, we note that our data could reflect variation among paralogs within and among infections, although we found no direct evidence for this. However, such intra-individual diversity is almost certainly lower than diversity between hosts, such that it is unlikely to have confounded the overall patterns we describe.

In our study area, several primates frequently raid crops, with the most common offenders being baboons, red-tailed guenons, and chimpanzees (Naughton-Treves *et al.* 1998; Naughton 1998; Mackenzie & Ahabyona 2012). Such interactions facilitate the transmission of gastrointestinal bacteria, protozoa and helminths in the Kibale system (Goldberg *et al.* 2007; Salzer *et al.* 2007; Goldberg *et al.* 2008c; Johnston *et al.* 2010; Salyer *et al.* 2012; Ghai *et al.* 2014). Although these interactions make cross-species transmission ecologically plausible, it remains unclear why one *Trichuris* lineage appears able to cross species boundaries with apparent ease, yet another other clades show host affinity (Group 1).

In conclusion, our phylogenetic analysis suggests that *Trichuris* is not a single species, but a species complex (see also Nissen *et al.* (2012) and Liu *et al.* (2013)) of co-circulating clades that

includes *T. suis*. Despite being sympatric, different clades appear to have different host affinity. Group 1 was specific to humans in our study, Group 2 has an intermediate host range, and Group 3 appears capable of infecting all primates sampled, including humans. While our analyses do not indicate whether Group 3 *Trichuris* is transmitted from primates to humans or *vice versa*, they do show that certain lineages within the *Trichuris* taxonomic complex should be considered multi-host pathogens, at least within the order Primates. Our results also demonstrate that *Trichuris* is among the 20% of helminths capable of cross-infecting primates and humans. Taxonomic and epidemiological studies of other soil-transmitted helminths in wild primates, many of which cause "neglected" tropical diseases (Hotez *et al.* 2008), may reveal yet more helminth taxa to be multi-host pathogens. If so, this would challenge past assumptions about the host specificity of helminth parasites while raising new concerns about global human and animal health.

Table 3.1: Prevalence of *Trichuris*. Sample size and prevalence of *Trichuris* spp. from nine host species (including humans) in and near Kibale National Park, Uganda based on microscopy and PCR of ITS1 and ITS 2 rDNA genes. Host species codes: BM = blue monkey, BW = black-and-white colobus, CH = chimpanzee, GM = grey-cheeked mangabey, HU = human, LM = l'hoest monkey, OB = olive baboon, RC = red colobus, and RT = red-tailed guenon.

		Total no. *Trichuris* Positive		Prevalence (95% CI)		No. Sequenced	
Species	N	Microscopy	PCR	Microscopy	PCR	ITS1	ITS2
BM	33	9	9	27.3 (15-44)	27.3 (15-44)	5	5
BW	37	12	13	32.4 (20-49)	35.1 (22-51)	9	9
CH	30	4	4	13.3 (5-30)	13.3 (5-30)	3	1
GM	42	6	6	14.3 (6-28)	14.3 (6-28)	4	1
HU	36	8	11	22.2 (11-38)	30.6 (18-47)	9	4
LM	8	5	5	62.5 (30-87)	62.5 (30-87)	4	2
OB	27	24	24	88.9 (71-97)	88.9 (71-97)	8	3
RC	64	15	15	23.4 (15-35)	23.4 (15-35)	9	5
RT	41	21	22	51.2 (36-66)	53.7 (39-68)	11	5
TOTAL	318	104	108	32.7 (28-38)	34.0 (29-39)	62	35

Table 3.2: Genetic differences between lineages of *Trichuris*, by host species. Below diagonal: pairwise nucleotide differences per site averaged across all sequence pairs, with standard errors (calculated from 1000 bootstrap replicates) in parentheses. Above diagonal: *Trichuris* lineage differentiation between hosts (PhiPT; an analog of F_{ST}), with probability values based on 999 permutations. Significant values ($P<0.05$) generated from 999 permutations are indicated with asterisks. Within diagonal: within-group nucleotide substitutions per site between all sequences within a host species. Host species abbreviations are: BM = Blue monkey, BW = Black-and-white colobus, CH = Chimpanzee, LM = L'hoest monkey, OB = Olive baboon, RC = Red colobus, RT = Red-tailed guenon, and HU = Human.

	BM	BW	CH	LM	OB	GM	RC	RT	HU
BM	***0.001***	0.000	0.000	0.000	0.214	0.127	0.000	0.040	0.053
BW	0.025 (0.003)	***0.030***	0.032	0.052	0.227*	0.157	0.013	0.118	0.105
CH	0.002 (0.001)	0.032 (0.004)	***0.002***	0.000	0.000	0.000	0.000	0.000	0.000
LM	0.001 (0.000)	0.033 (0.004)	0.001 (0.001)	***0.000***	0.000	0.000	0.000	0.000	0.000
OB	0.007 (0.002)	0.041 (0.004)	0.009 (0.002)	0.008 (0.002)	***0.013***	0.000	0.034	0.000	0.000
GM	0.001 (0.000)	0.037 (0.004)	0.002 (0.001)	0.001 (0.000)	0.009 (0.002)	***0.001***	0.000	0.000	0.000
RC	0.008 (0.001)	0.030 (0.003)	0.010 (0.001)	0.009 (0.001)	0.017 (0.002)	0.010 (0.001)	***0.017***	0.000	0.000
RT	0.001 (0.000)	0.033 (0.004)	0.002 (0.001)	0.001 (0.000)	0.009 (0.002)	0.002 (0.000)	0.010 (0.001)	***0.002***	0.000
HU	0.063 (0.040)	0.088 (0.043)	0.049 (0.038)	0.053 (0.004)	0.058 (0.004)	0.048 (0.004)	0.061 (0.034)	0.052 (0.012)	***0.090***

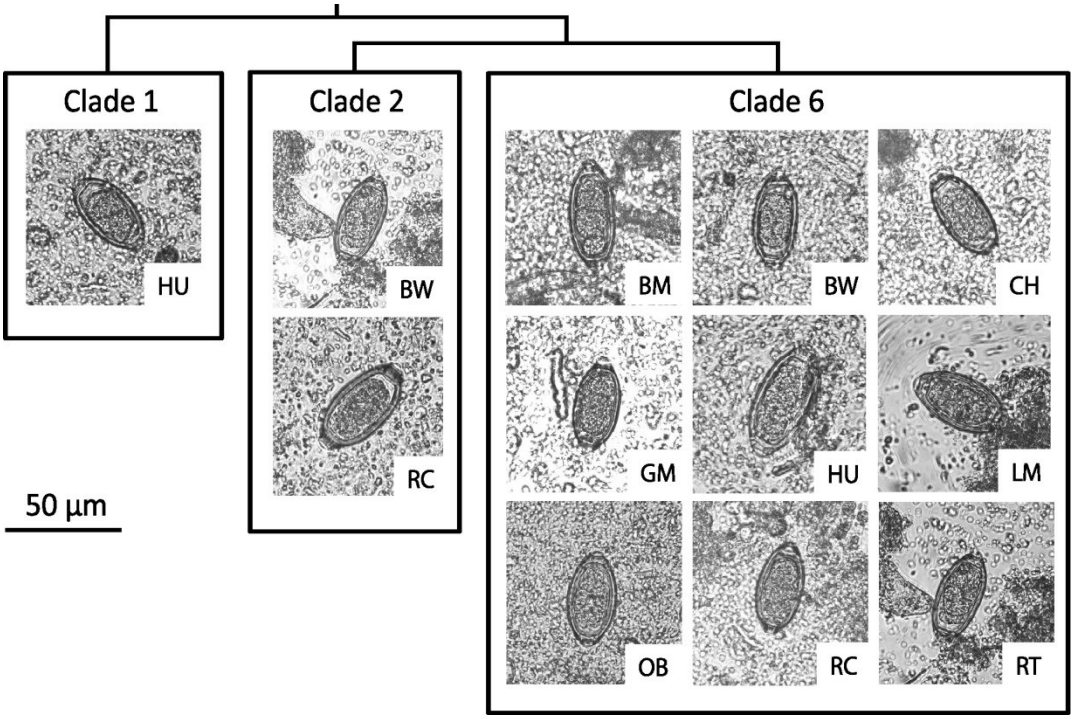

Figure 3.1: Representative *Trichuris* eggs from nine hosts. *Trichuris* eggs were photographed at 40X objective magnification. *Trichuris* eggs were identified in thin smears of sedimented feces from infected hosts. Images demonstrate considerable morphological variation in egg size and shape (50-76 µm in length, 26-30 µm in width), although differences in the ratio of length to width among parasite clades and among host species were not significant (Kruskall-Wallis tests, $P > 0.05$). The cladogram on the top of the figure is a simplified version of the phylogenetic tree shown in Figure 3.2 and represents the relative relatedness of *Trichuris* clades. Host species abbreviations follow Table 3.2.

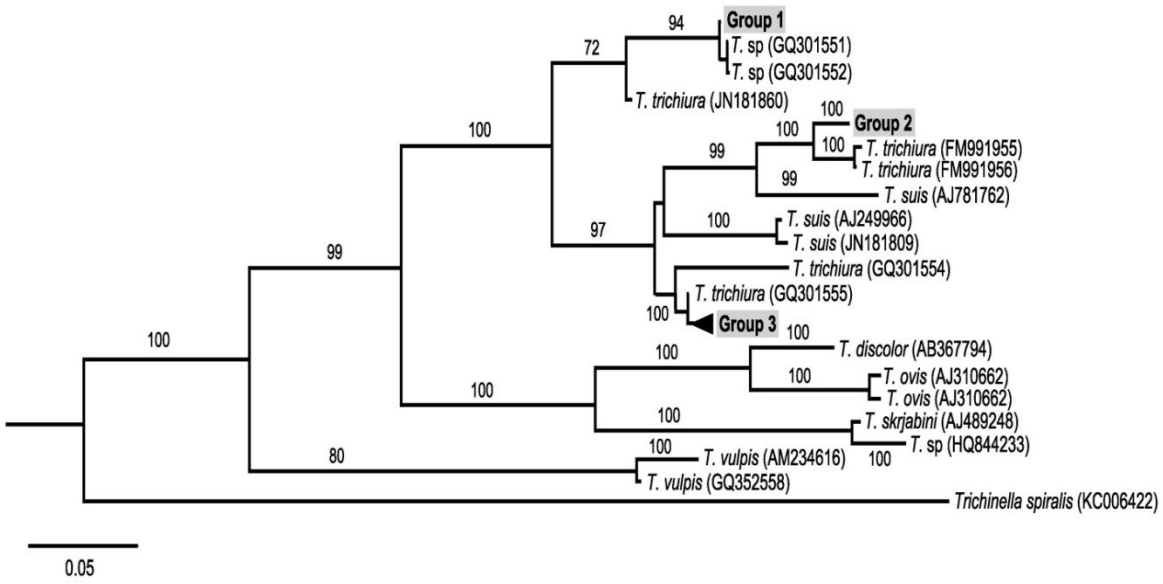

Figure 3.2: Phylogenetic tree of *Trichuris*. Bayesian phylogenetic tree of *Trichuris* based on concatenated ITS 1 and ITS 2 rDNA sequences. 18S, ITS 1 (895 bp), and 5.8S, ITS 2, 28S (486 bp) sequences were concatenated, and regions of ambiguous alignment removed in Gblocks. Phylogenetic relationships were inferred using MrBayes, with newly generated sequences clustering in three groups: Group 1 (2 human samples), Group 2 (4 black-and-white colobus and 1 red colobus samples), and Group 3 (7 blue monkey, 9 black-and-white colobus, 4 chimpanzee, 8 human, 4 grey-cheeked mangabey, 4 l'hoest monkey, 8 olive baboon, 10 red colobus, and 12 red-tailed guenon samples). Posterior clade probabilities are shown next to branches. Reference sequences (*T. trichiura*, *T suis*) and outgroups (*T. discolor*, *T. ovis*, *T. skrjabini*, *T. sp*, *T. vulpis* and *Trichinella spiralis*) are italicized, with GenBank accession numbers included in parenthesis. Scale bar indicates nucleotide substitutions per site.

LINKING STATEMENT 2

In the previous chapter, I examined the host range of a second soil transmitted helminth, *Trichuris*, within the primate community and adjacent human population. I discovered three lineages of *Trichuris*. Two of these lineages appeared host specific, as they were only found in humans or colobines. The third lineage comprised over 90% of all sequenced samples, and was found in all species of primate included in this research.

These results do not strictly adhere to expectations that soil transmitted helminths would be host specialists (Pedersen *et al.* 2008). Two lineages of *Trichuris* appeared limited in host range, which agree with my predictions of host specialization among soil transmitted helminths. However, both these lineages are found in other primate species elsewhere, and thus may not be true specialists, just uncommon variants in this community. The third lineage was found in all primates, which indicates host generalism, at least within primates.

Neither phylogeny nor primate host traits predicted *Trichuris* distribution or prevalence (an NRI analysis was conducted to identify phylogenetic structure of *Trichuris* lineages, but did not return significant results and was removed from the manuscript). Why two *Trichuris* lineages seem to be host specific and another generalist within this community remains unanswered.

Both *Trichuris* and *Oesophagostomum* contained broadly transmissible lineages. These results are surprising, since previous research found that the majority of primate helminths are species-specific (Pedersen *et al.* 2005). However, parasites transmitted indirectly (environment and vector) such as these will frequently encounter many potential host species (Woolhouse *et al.* 2001). If adaptation to hosts is not as limiting as proposed, the probability of encountering multiple hosts species might be the best predictor of host range, which fits my observations.

In the next chapter, I will examine the host range and transmission of the blood parasites *Plasmodium* and *Hepatocystis* – which have a very different mode of transmission and life history than soil transmitted helminths. By comparing two similar parasites (soil transmitted helminths) with two dissimilar parasites, I will be able to examine how parasite traits influence host range and transmission, while still keeping the primate community constant.

CHAPTER 4: PART I

Co-infection and cross-species transmission of divergent *Hepatocystis* lineages in a wild African primate community

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4.1 Abstract

4.1.1 Background

Hemoparasites of the apicomplexan family Plasmodiidae include the etiological agents of malaria, as well as a suite of non-human primate parasites from which the human malaria agents evolved. Despite the significance of these parasites for global health, little information is available about their ecology in multi-host communities. Here, we investigate the transmission of blood parasites from the genera *Plasmodium* and *Hepatocystis* circulating in a wild community of primates.

4.1.2 Methods and Findings

Primates were investigated in Kibale National Park, Uganda, where ecological relationships among host species are well-characterized. Blood samples were examined for *Plasmodium* and *Hepatocystis* using microscopy and PCR targeting the parasite mitochondrial cytochrome *b* gene, followed by Sanger sequencing. To assess co-infection, ‘‘deep sequencing’’ of a variable region within cytochrome *b* was performed. Out of nine black-and-white colobus (*Colobus guereza*), one blue monkey (*Cercopithecus mitis*), five grey-cheeked mangabeys (*Lophocebus albigena*), 23 olive baboons (*Papio anubis*), 52 red colobus (*Procolobus rufomitratus*) and 12 red-tailed guenons (*Cercopithecus ascanius*), 79 infections (77.5%) were found, all of which were *Hepatocystis* spp. Sanger sequencing revealed 25 different parasite haplotypes that phylogenetically sorted into six species-specific but morphologically similar lineages. ‘‘Deep sequencing’’ revealed mixed-lineage co-infections in baboons and red colobus (41.7% and 64.7% of individuals, respectively) but not in other host species. One lineage infecting red colobus also infected baboons, but always as the minor variant, suggesting directional cross-species transmission.

4.1.3 Conclusions

Hepatocystis parasites in this primate community are a diverse assemblage of cryptic lineages, some of which co-infect hosts and at least one of which can cross primate species barriers. The species-specificity evident from our results suggest that host factors, rather than geography, predict transmission in this ecologically important family of parasites.

4.2 Introduction

Hemoparasites of the apicomplexan family Plasmodiidae include some of the most globally important human pathogens. Over 20 species of *Plasmodium*, which cause malaria, are known to infect non-human primates and at least four species, *Plasmodium inui, Plasmodium simium, Plasmodium cynomolgi* and *Plasmodium knowlesi* are known to be transmissible to humans (Coatney *et al.* 2003; Cox-Singh *et al.* 2008). Human malaria parasites, including the particularly virulent *Plasmodium falciparum*, have zoonotic origins in non-human primates (Escalante *et al.* 1998; Rich *et al.* 2009; Duval *et al.* 2010; Krief *et al.* 2010a; Liu *et al.* 2010; Prugnolle *et al.* 2011). However, the specific primate species from which each human malaria agent originated remains a matter of debate. Although the closest relative of *P. falciparum* was previously considered to be *Plasmodium reichenowi* which is found in chimpanzees (*Pan troglodytes*), recent studies have discovered more closely related *Plasmodium* variants in other African apes and monkeys (Rich *et al.* 2009; Duval *et al.* 2010; Krief *et al.* 2010a; Liu *et al.* 2010; Prugnolle *et al.* 2011).

Studies of *Plasmodium* and its relatives in non-human primates have been highly informative but influenced by the geography of sampling. Samples from captive animals close to humans may bias results towards zoonotic and reverse zoonotic transmission (Liu *et al.* 2010; Prugnolle *et al.* 2011). Broad surveys of primate hosts have yielded valuable data on geographic patterns of infection, but inferences about local transmission are difficult to make from such studies (Liu *et al.* 2010; Ayouba *et al.* 2012). Focused investigations of particular host species are useful for measuring local prevalence and parasite diversity but less so for examining transmission among species (Krief *et al.* 2010a; Liu *et al.* 2010). Evaluating the propensity for *Plasmodium* and its relatives to cross species barriers in nature would require studying well-defined ecological communities where multiple host species are potentially exposed to the same vector populations (Prugnolle *et al.* 2011).

This study reports results from the well-characterized primate community of Kibale National Park, Uganda (hereafter Kibale) and the infection of its constituent host species with parasites of the genera *Plasmodium* and *Hepatocystis*. Like *Plasmodium*, *Hepatocystis* is a hemosporidian parasite within the family Plasmodiidae that infects primates (Desser & Bennett 1993). Unlike

Plasmodium, *Hepatocystis* is transmitted by biting midges of the genus *Culicoides,* rather than *Anopheles* mosquitoes (Garnham 1966). At least four species of *Hepatocystis* are known to infect African monkeys: *Hepatocystis kochi, Hepatocystis simiae, Hepatocystis bouillezi* and *Hepatocystis cercopitheci* (Seethamchai et al. 2008). *Hepatocystis* can cause parasitemia for up to 15 months without causing clinical signs (Vickerman 2005), although early studies documented anemia and visible merocyst production, followed by scarring of the liver (Garnham 1966). Primates can be infected repeatedly and prevalence increases with age (Vickerman 2005). *Hepatocystis* is not known to infect humans.

Microscopy and molecular methods were used to assess infection in six species of monkeys in Kibale: black-and-white colobus (*Colobus guereza*), blue monkeys (*Cercopithecus mitis*), grey-cheeked mangabeys (*Lophocebus albigena*), olive baboons (*Papio anubis*), red colobus (*Procolobus rufomitratus*), and red-tailed guenons (*Cercopithecus ascanius*). ‘‘Deep sequencing’’ was then used to assess co-infection of individual hosts with multiple parasite variants. To our knowledge, this is the first study of cross-species transmission of malaria-like parasites in a well-defined community of wild primates where host species overlap spatially and temporally and would thus encounter the same vector population.

4.3 Materials and Methods

4.3.1 Study Site and Sampling

Kibale is a 795 km^2 semi-deciduous, mid-altitude forest in western Uganda (0°130 –0°410 N, 30°190 –30°320 E) (Chapman & Lambert 2000) within the Albertine Rift, an area of exceptional biological diversity and conservation value (Plumptre *et al.* 2007). Kibale’s primate community has been studied for over 40 years, such that a wealth of information exists on the ecology of its 13 constituent species (Chapman *et al.* 2005b). *Plasmodium* infection has previously been documented in Kibale’s chimpanzees (Krief *et al.* 2010a).

As part of a larger study of primate ecology, conservation and health (Goldberg *et al.* 2012), 34 animals in 2006 and 68 animals in 2010 were chemically immobilized as previously described (Goldberg *et al.* 2009; Lauck *et al.* 2011). Blood was drawn from the femoral vein, placed into an evacuated plasma collection tube (Becton, Dickinson and Company, Inc, Franklin Lakes, NJ,

USA) and kept cool until processing. At the time of sampling, two thin blood smears were prepared, which were fixed and stained with Giemsa within 5 hours of collection. Blood was separated by centrifugation in a field laboratory and frozen immediately in liquid nitrogen for storage and transport. Samples were shipped following International Air Transport Association (IATA) regulations under Ugandan Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) permit #002290.

4.3.2 Microscopy

Thin blood smears were viewed under 1000x oil immersion using a Nikon Eclipse e600 light microscope. For each slide, a field containing a representative monolayer of red blood cells (RBCs) was identified and all RBCs in that field were counted. This process was repeated five times, to produce an average number of RBCs per field. One hundred fields, or the maximum number of viewable fields on the slide, were then scanned for intra-erythrocytic parasites morphologically consistent with *Plasmodium* or *Hepatocystis*. Intensity of infection was calculated as (Warhurst & Williams 1996) :

$$\frac{\text{Total number of infected RBCs}}{(\text{Average number of RBCs per field} \times \text{Number of fields scanned}) \times 100}$$

4.3.3 PCR and Sequencing

DNA was extracted from blood using a Zymo Quick-gDNA Mini-Prep kit (Zymo Research, Irvine, CA, USA) according to the manufacturer's instructions. PCR targeting the mitochondrial cytochrome *b* gene of *Plasmodium* and *Hepatocystis* was conducted using newly designed ''universal'' primers CytB3384F (5'-GTAATGCCTAGACGTATTCCTG-3') and CytB4595R (5'-GTTTGCTTGGGAGCTGTAATC-3'), which generate amplicons of predicted sizes 1,391 bp for *Hepatocystis* spp. and 1,254 bp for *Plasmodium* spp. PCRs were performed in 25 µl volumes using the FailSafe system (EpiCenter Biotechnologies, Madison, WI, USA), with reactions containing 1x FailSafe PCR PreMix with Buffer E, 1 Unit of FailSafe Enzyme Mix, 2.5 pmol of each primer and 1 µl of template. Reactions were cycled in a Bio-Rad CFX96 platform (Bio-Rad Laboratories, Hercules, CA, USA) with the following temperature profile: 94 °C for 1 min; 45 cycles of 94 °C for 15 sec, 50 °C for 30 sec, 72 °C for 90 sec; and a final extension at 72

°C for 10 min. Amplicons were electrophoresed on agarose gels stained with ethidium bromide and purified from gels using the Zymoclean Gel DNA Recovery Kit (Zymo Research, Irvine, CA, USA) according to the manufacturer's instructions.

Products were Sanger sequenced in both directions using primers CytB3384F and CytB4595R, as well as internal sequencing primers CytB4081F (5'-TTACATTTACATGGTAGCAC-3') and Cytb4062R (5'-GTGCTACCATGTAAATGTAA-3') on ABI 3730xl DNA Analyzers (Applied Biosystems, Carlsbad CA, USA) at the University of Wisconsin Biotechnology Center DNA Sequence Facility. Sequences were hand-edited using Sequencher v4.9 (Gene Codes Corporation, Ann Arbor MI, USA) and all ambiguous bases were resolved by repeat PCR and re-sequencing, as described above. All new sequences were deposited in GenBank, under Accession Nos. KC262794 - KC262867.

4.3.4 Pyrosequencing

To assess co-infection, 40 samples were chosen for multiplexed pyrosequencing based on phylogenetic analyses of Sanger sequences, such that all combinations of lineages and host species were represented. The primers 454cytbF (5'-GAGAATTATGGAGTGGATGGTGTT-3') and 454cytbR (5'-ATGCTGTATCATACCCTAAAGGATT-3') were designed to amplify 420 bp of a variable and phylogenetically informative region of cytochrome *b* within H*epatocystis* sequences from Kibale, based on analysis of Sanger sequences. To allow multiplexing, unique identification tag sequences (10 bp) and adaptor sequences (25 bp) were designed for each of the 40 individuals. PCRs were conducted with the Phusion High-Fidelity PCR Kit (Finnzymes, Espoo, Finland) in 20 µl volumes containing 1X Phusion HF buffer, 10 mmol of dNTPs, 1 unit of Phusion DNA Polymerase, 5.0 pmol of each primer and 1 µl of original DNA extract as template. Reactions were cycled in a Bio-Rad CFX96 platform (Bio-Rad Laboratories, Hercules, CA, USA) with the following temperature profile: 98 °C for 30 sec; 30 cycles of 98 °C for 10 sec, 57 °C for 15 sec, 72 °C for 150 sec; and a final extension at 72 °C for 5 min. Amplicons were purified as described above (Section 2.3) and further cleaned using the AMPure XP PCR purification system (Beckman Coulter, Brea CA, USA). DNA was quantitated using the Quant-iT dsDNA HS Assay kit (Life Technologies, Madison, WI, USA), concentrations were normalized to the least concentrated sample, and samples were pooled at

$2x10^6$ molecules/µl. Pooled samples were then subjected to 454 emulsion PCR, and products were pyrosequenced on a GS Junior instrument (Roche 454 Life Sciences, Branford, CT, USA).

4.3.5 Statistical Analyses

Frequencies of infection and co-infection were calculated as simple ratios, with 95% confidence limits estimated using the modified Wald method (Agresti & Coull 1998). Mean and median intensities of infection were calculated and 95% confidence limits were generated using the computer program Quantitative Parasitology 3.0 (Rozsa *et al.* 2000), which was also used to perform Mood's median tests (Mood 1954) to compare intensities of infection among groups. Frequencies of infection among groups were compared using Fisher's exact tests and other statistical comparisons were performed using the computer program SPSS Statistics 17.0 (SPSS, Inc., Chicago, IL, USA).

Sanger sequences were aligned using the computer program ClustalX (Larkin *et al.* 2007) with minor manual adjustment. To construct phylogenetic trees, the neighbor joining method was applied (Saitou & Nei 1987) using the computer program MEGA5 (Tamura *et al.* 2011), with a maximum composite likelihood distance correction and 1,000 bootstrap replicates of the data to assess robustness of phylogenetic groupings. Phylogenetic trees were constructed both from full-length alignments of newly generated *Hepatocystis* sequences (1,178 positions) and from alignments trimmed to 726 bp to allow inclusion of shorter published reference sequences (Ayouba *et al.* 2012).

To assess co-infection of individual primates with multiple *Hepatocystis* lineages using pyrosequencing data, individual sequences from each animal were mapped to newly generated and published *Hepatocystis* cytochrome *b* sequences using CLC Genomics Workbench 5.1 (CLC Bio, Aarhus, Denmark) with stringent settings, including minimum 95% similarity, 50% length fraction, and insertion and deletion costs of three. Sequences were mapped to each animal's Sanger consensus sequence, to all other *Hepatocystis* variants identified in Kibale primates as described above, and to *Hepatocystis* reference sequences available in GenBank. An individual was considered to be co-infected when at least 5% of sequences within that individual that mapped to a *Hepatocystis* lineage other than the lineage defined by that animal's Sanger

consensus sequence. To guard against false positive results, a filtering step was applied to exclude low quality and truncated sequences (less than 300 bases).

4.4 Results

4.4.1 Microscopy and PCR

Microscopy identified intra-erythrocytic parasites in 75% of blood smears across six primate species in Kibale (Table 4.1). Parasites displayed trophozoite and gametocyte forms that were morphologically similar in all host species (Fig. 4.1). Prevalence ranged from 22.2% in black-and-white colobus to 91.3% in baboons (Table 4.1). Mean intensity (percentage of infected red blood cells) across all species was 0.058%, ranging from 0.052% in baboons to 0.090% in black-and-white colobus. PCR yielded amplicons of the predicted size in 77.5% of blood DNA extracts across all host species, with species-specific prevalence agreeing closely with microscopy (Table 4.1). Based on both microscopy and PCR, prevalence varied significantly across the four host species in which infection was detected (Table 4.1; Fisher's exact $p < 0.001$ in both cases). However, intensity was only marginally different across the same four host species (Table 4.1; Mood's exact $p = 0.059$).

4.4.2 Sanger Sequences

Analysis of Sanger sequences revealed 25 *Hepatocystis* haplotypes within the 79 PCR-positive Kibale primate samples. No primates were infected with *Plasmodium*. Phylogenetic analysis sorted these haplotypes into six host-specific lineages, arbitrarily numbered 1–6 (Fig. 4.2A). Lineage 1 infected baboons, lineages 2 and 3 infected red colobus, lineages 4 and 6 infected red-tailed guenons, and lineage 5 infected black-and-white colobus. Bootstrap values indicated strong to moderate support for this pattern of clustering. Intensity of infection (measured using microscopy; see above) was not statistically different across the six lineages (Table 4.1; Mood's exact $p = 0.100$).

A second phylogenetic tree was generated using sequences trimmed to 726 bp to incorporate 16 unique published *Hepatocystis* reference sequences (Escalante *et al.* 1998; Prugnolle *et al.* 2011; Ayouba *et al.* 2012). All six species-specific lineages identified in Fig. 4.2A were retained in this second phylogeny (Fig. 4.2B). However, the position of lineage 6 shifted, due to the exclusion of

a region at the 30 end of the full-length alignment within which lineage 6 contained several point substitutions and a deletion. A reference sequence from an Ethiopian baboon (*Papio* sp.) clustered within lineage 1, which infects Kibale baboons, and a reference sequence from a greater spot-nosed guenon (*Cercopithecus nictitans*) was identical to a haplotype within lineage 3, which infects Kibale red colobus. Other reference sequences from greater spot-nosed guenons, mustached guenons (*Cercopithecus cephus*), a mandrill (*Mandrillus sphinx*), and a talapoin (*Miopithecus talapoin*) clustered separately from the six Kibale lineages but fell within the overall diversity circumscribed by the Kibale lineages.

4.4.3 Pyrosequencing and Co-Infection

Multiplexed pyrosequencing of 40 *Hepatocystis* 420 bp amplicons yielded an average of 646 high-quality sequences per individual (S.D. = 232; range 278–1,198) of average length 398 bp (S.D. = 25; range 302–420 bp). Mapping individual sequences to Sanger sequences from each Kibale primate and published reference sequences identified mixed-lineage co-infections in 21 of 40 individuals (52.5%). All co-infections occurred in either baboons (5/12; 41.7%) or red colobus (11/17; 64.7%). There was no evidence of co-infections in red-tailed guenons ($n = 9$) or black-and-white colobus ($n = 2$). Frequencies of mixed-lineage co-infection were statistically different across host species (Fisher's exact $p = 0.004$). The number of sequences within singly infected individuals (mean = 701 ± 265) and co-infected individuals (mean = 564 ± 141) did not differ statistically (Mann–Whitney $U = 153.5$; $z = 1.05$; $p = 0.294$), demonstrating that assessment of co-infection was not biased by variation in sequencing ''depth'' among individuals.

Fig. 4.3 shows the pattern of single lineage and mixed lineage infection and co-infection, respectively, for the 40 individuals subjected to pyrosequencing. Black-and-white colobus were singly infected only with lineage 5. Red-tailed guenons were singly infected with either lineage 4 (1/9; 11.1%) or lineage 6 (8/9; 88.9%). Red colobus were either singly infected with lineage 2 (4/17; 23.5%) or lineage 3 (2/17; 11.8%), or co-infected with a mixture of lineages 2 and 3 (11/17; 64.7%). The ratio of lineage 2: lineage 3 sequences within the 11 co-infected red colobus ranged from 1:8.5 to 7.7:1. Baboons were either singly infected with lineage 1 (7/12; 58.3%) or co-infected with a mixture of lineages 1 and 2 (5/12; 41.7%). In co-infected baboons, lineage 2

was always the minor variant; the ratio of lineage 1: lineage 2 sequences within the five co-infected baboons ranged from 14.4:1 to 4.6:1. There was no evidence of co-infection of any Kibale primate with any *Hepatocystis* lineage that was not detected in the primates of Kibale using Sanger sequencing.

4.5 Discussion

This study examined a community of wild primates in Uganda where ecological relationships among host species are well defined and where spatio-temporal overlap would make cross-species transmission of vector-borne parasites possible. Microscopy and PCR indicated rates of infection with *Hepatocystis* ranging from approximately 20% to 90% across host species. This finding is consistent with published data from wild greater spot-nosed guenons in Cameroon, which documented a prevalence of *Hepatocystis* infection of 49% (Ayouba *et al.* 2012). Even though *Hepatocystis* infection was common, there was no evidence of *Plasmodium* infection, despite the fact that *Plasmodium* infects chimpanzees from the same location (Krief *et al.* 2010a). These results suggest that primate hosts may vary in their susceptibility to parasites of the family Plasmodiidae, even when those hosts live in the same environment.

Despite morphological similarity among parasites, phylogenetic analyses of cytochrome *b* sequences yielded strong evidence that multiple, host-specific *Hepatocystis* lineages infect Kibale primates (Fig. 4.2A). These results are in contrast to the aforementioned study of Cameroonian greater spot-nosed guenons, which showed a lack of phylogenetic clustering of *Hepatocystis* haplotypes infecting this species (Ayouba *et al.* 2012). However, the results of this study are consistent with findings of host specificity in parasites in the sub-genus *Laverania* infecting wild chimpanzees and lowland gorillas (*Gorilla gorilla*) also from eastern and central Africa (Liu *et al.* 2010). The current findings may have resulted from the longer cytochrome *b* sequences generated in this study, relative to previously published methods (Liu *et al.* 2010). However, sequences were trimmed to allow comparison with shorter published reference sequences (Fig. 4.2B), the general pattern from the analysis of full-length sequences persisted.

These results suggest that the evolution of the *Hepatocystis* genus is shaped principally by host factors, rather than by geography. Kibale, a forest park of only 795 km^2, contains as much

Hepatocystis cytochrome *b* phylogenetic diversity as has previously been documented in primates across sub-Saharan Africa (Prugnolle *et al.* 2011; Ayouba *et al.* 2012). The Albertine Rift wherein Kibale lies is a reservoir of biological diversity (Plumptre *et al.* 2007); perhaps the diversity of *Hepatocystis* lineages within Kibale reflects the unique biogeography of this region. It is also noteworthy that one published reference sequence from an Ethiopian baboon clusters within Kibale lineage 1, which infects olive baboons, a related species (Fig. 4.2B), again suggesting a strong role of host factors in shaping *Hepatocystis* evolution.

To our knowledge, this is the first study to use ‘‘deep sequencing’’ to investigate co-infection and cross-species transmission in a malaria-like parasite. Pyrosequencing of a portion of cytochrome *b* showed that mixed-lineage co-infection is common in Kibale red colobus and baboons. No mixed-lineage co-infections were detected in red-tailed guenons, despite roughly equal sampling intensity. The ability to detect mixed-lineage co-infections in black-and-white colobus was limited by the small number of infected individuals. These results complement a previous study of Asian crab-eating macaques (*Macaca fasicularis*), showing that one animal out of 99 was infected with two distinct *Hepatocystis* clones (Seethamchai *et al.* 2008).

In red colobus, individuals were singly and dually infected with two *Hepatocystis* lineages. Within co-infected red colobus, some individuals were co-infected with approximately equal proportions of both lineages. In contrast, there were a lower proportion of co-infected baboons, and all such baboons were co-infected with one of the red-colobus-associated lineages as the minor variant. Furthermore, no baboon was singly infected with this same red colobus-associated lineage. Thus, despite co-infection of baboons with red colobus-associated *Hepatocystis,* no red colobus were co-infected with the corresponding baboon-associated lineage. These results imply that red colobus-associated *Hepatocystis* can cross primate species barriers to infect baboons, but that baboon-associated *Hepatocystis* does not show a similar pattern of transmission in the other direction.

Unfortunately, little information exists on the vectors that transmit these parasites in Kibale. *Hepatocystis* is thought to be transmitted by biting midges of the genus *Culicoide*s (Garnham et al. 1961). The patterns of infection observed for *Hepatocystis* in Kibale primates could reflect the

distribution and/or differential host feeding preferences of vectors. Clearly, investigations of midges and other potential insect vectors in Kibale are in order, perhaps coupled with blood meal analyses to identify patterns of host preference (Molaei & Andreadis 2006; Lassen *et al.* 2011). Primates in Kibale often form mixed species groups, also known as polyspecific associations, perhaps to guard against predation (Chapman & Chapman 1996). In such groups, red colobus associate preferentially with red-tailed guenons, and less so with black-and-white colobus (Chapman & Chapman 2000b). Baboons are largely terrestrial and do not form polyspecific associations with these three arboreal species. The patterns of cross-species transmission between red colobus and baboons described herein cannot therefore be explained by patterns of association among host species in Kibale.

It should be noted that a conservative analysis of pyrosequencing data may have underestimated rates of mixed-lineage co-infection. For example, small numbers of truncated, low quality sequences were found in additional baboons and red colobus consistent with the patterns shown in Fig. 4.3; these would have increased estimates of the frequency of co-infection had they been included. Haplotype-specific PCR followed by Sanger sequencing may have higher sensitivity than pyrosequencing for detecting mixed-lineage co-infection, although perhaps with higher error rates. With improvements in ‘‘next generation’’ sequencing technologies, it should be possible to identify very low levels of mixed lineage co-infection using methods similar to those described herein (Shokralla *et al.* 2012), and without the need for labour-intensive cloning (Seethamchai *et al.* 2008). It should also be noted that sequence-level mitochondrial heteroplasmy has been documented in certain parasite taxa (e.g. Bowles *et al.* (1995), van Herwerden *et al.* (2000), Curtis *et al.* (2001), Le *et al.* (2002), Messenger *et al.* (2012)). However, the host-specific and asymmetrical patterns of infection documented for *Hepatocystis* in Kibale primates are inconsistent with this type of variation, as well as with the low levels of random genetic variation that would be expected from processes such as mutation or sequencing error.

Overall, the results of this study show that *Hepatocystis* exists in the Kibale primate community as a diverse assemblage of cryptic lineages, some of which are capable of co-infecting hosts and at least one of which can cross primate species barriers. These observations, together with the

lack of *Plasmodium* infection in Kibale monkeys despite its presence in Kibale chimpanzees (Krief *et al.* 2010a), demonstrate that the cross-species transmission potential of the Plasmodiidae varies among parasite lineages, even when hosts live in the same environment. Understanding why certain parasites within the Plasmodiidae cross species barriers in nature could help predict which new or poorly studied members of this critically important parasite family are most likely to emerge.

Table 4.1: Prevalence and intensity of *Hepatocystis*. Sample size, prevalence and intensity of infection in six primate hosts from Kibale National Park, Uganda. Hosts: BM = blue monkey (*Cercopithecus mitis*), BW = black-and-white colobus (*Colobus guereza*), GM = grey-cheeked mangabey (*Lophocebus albigena*), OB = olive baboon (*Papio anubis*), RC = red colobus (*Procolobus rufomitratus*), RT = red-tailed guenon (*Cercopithecus ascanius*).

		Prevalence (%)[2]		Intensity (%)[3]	
Host	N[1]	Microscopy	PCR	Mean	Median
BW	9	22.2 (5.3 – 55.7)	22.2 (5.3 – 55.7)	0.090 (0.08 – 0.09)	0.090 (undefined)
BM	1	0 (0.0 – 83.3)	0 (0.0 – 83.3)	--	--
GM	5	0 (0.0 – 48.9)	0 (0.0 – 48.9)	--	--
OB	23	91.3 (72.0 – 98.8)	95.7 (77.3 – 99.9)	0.052 (0.03 – 0.09)	0.025 (95.7%: 0.01 – 0.03)
RC	46	89.1 (76.5 – 95.7)	88.5 (76.7 – 95.0)	0.058 (0.04 – 0.10)	0.038 (95.6%: 0.03 – 0.06)
RT	12	66.7 (38.8 – 86.5)	75.0 (46.2 – 91.7)	0.069 (0.04 – 0.12)	0.045 (96.1%: 0.01 – 0.16)
All	102	75.0 (65.4 – 82.6)	77.5 (68.4 – 84.5)	0.058 (0.05 – 0.08)	0.032 (95.6%: 0.02 – 0.04)

[1] Numbers indicate sample sizes of thin smears examined microscopically and blood DNA extracts used for PCR. Thin smears from six red colobus were not readable and were thus excluded.

[2] Numbers in parentheses are 95% confidence limits calculated using the modified Wald method

[3] Intensity was measured as the percent of infected red blood cells in positive animals, based on microscopic examination. Numbers in parentheses are bootstrap 95% confidence limits (mean) and "exact" confidence intervals (median), with the precise level of confidence around each median indicated (the confidence interval for black-and-white colobus was undefined because only two individuals were infected).

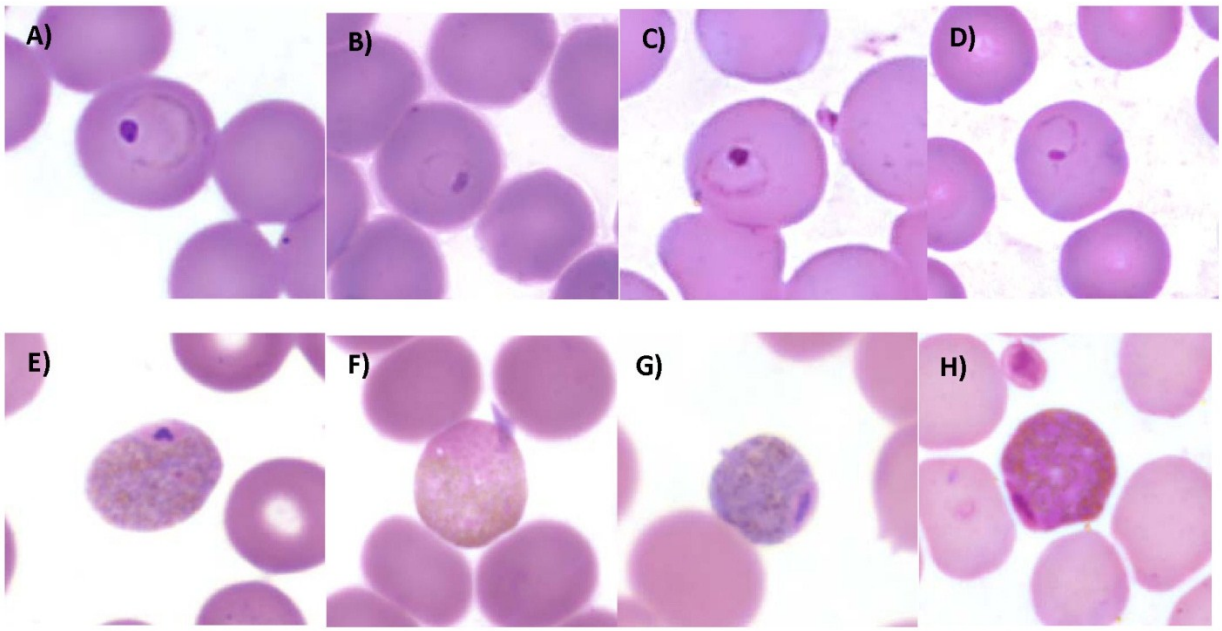

Figure 4.1: Ring and trophozoite stages of *Hepatocystis* in four primate host species. Ring stages (A-D) and trophozoite stages (E-F) were photographed at 1000X magnification in representative thin blood smears stained with 10% Giemsa for each of the four Kibale primate species positive for *Hepatocystis* (from left to right): olive baboon (*Papio anubis*), black-and-white colobus (*Colobus guereza*), red colobus (*Procolobus rufomitratus*), and red-tailed guenon (*Cercopithecus ascanius*).

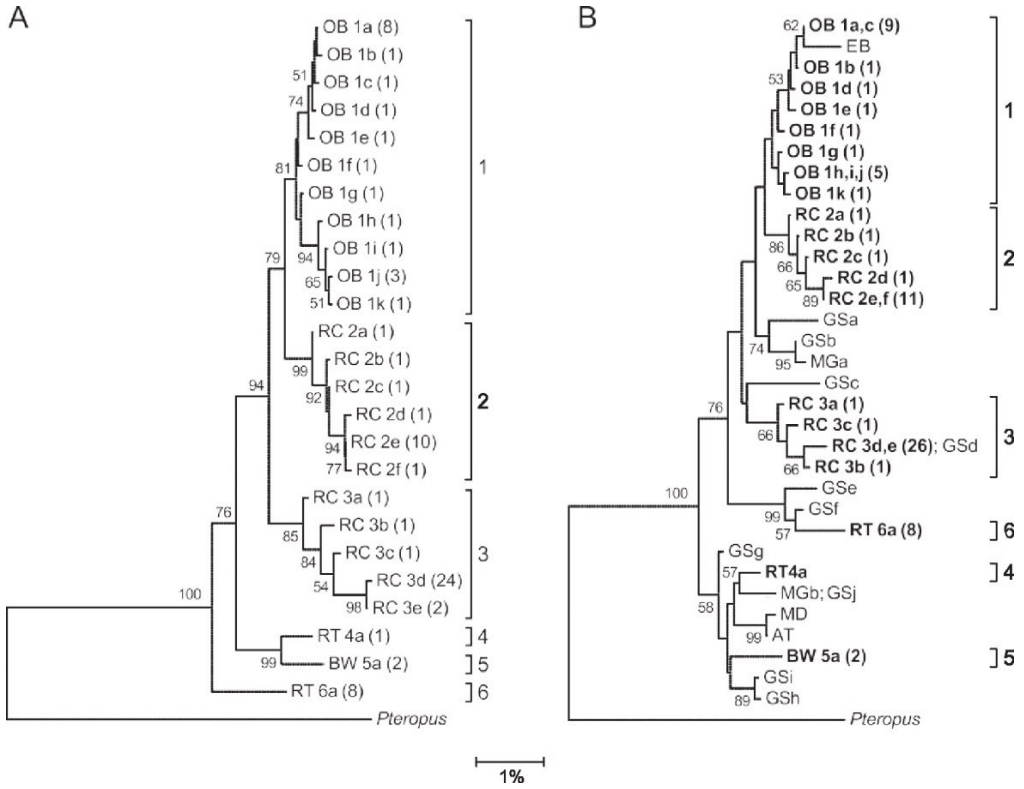

Figure 4.2: Phylogenetic trees of *Hepatocystis* spp. The tree in panel A was constructed from a 1,178-bp alignment of cytochrome b gene for all PCR-positive primates from Kibale. Numbers indicate bootstrap values (%), estimated from 1,000 re-samplings of the sequence data; bootstrap values ≤ 50% are not shown. Taxon names indicate the host species from which each sequence was recovered (OB = olive baboon; RC = red colobus; RT = red-tailed guenon; BW = black-and-white colobus), followed by an arbitrary haplotype designation and the number of individuals infected with that haplotype in parentheses. Brackets indicate species-specific lineages, arbitrarily labeled 1-6. The tree in panel B was constructed using identical methods, but with sequences trimmed to 726 bp to allow incorporation of shorter published reference sequences from an Ethiopian baboon (EB), 10 greater spot-nosed guenons (GSa-h), two mustached guenons (MGa,b), a mandrill (MD), and an Angolan talapoin (AT) (GenBank accession numbers AF069626, JF923757, JF923758, JF923759, JF923760, JQ070819, JQ070820, JQ070869, JQ070892, JQ070901, JQ070903, JQ070910, JQ070938, JQ070951, and JQ070953). Haplotypes sequenced in this study (bold type) follow the same naming convention as described above, and brackets indicate the same lineages. The trees are rooted with an outgroup *Hepatocystis* sequence from a small flying fox, *Pteropus hypomelanus* (GenBank accession number FJ168565). The scale bar indicates percent nucleotide substitutions per site.

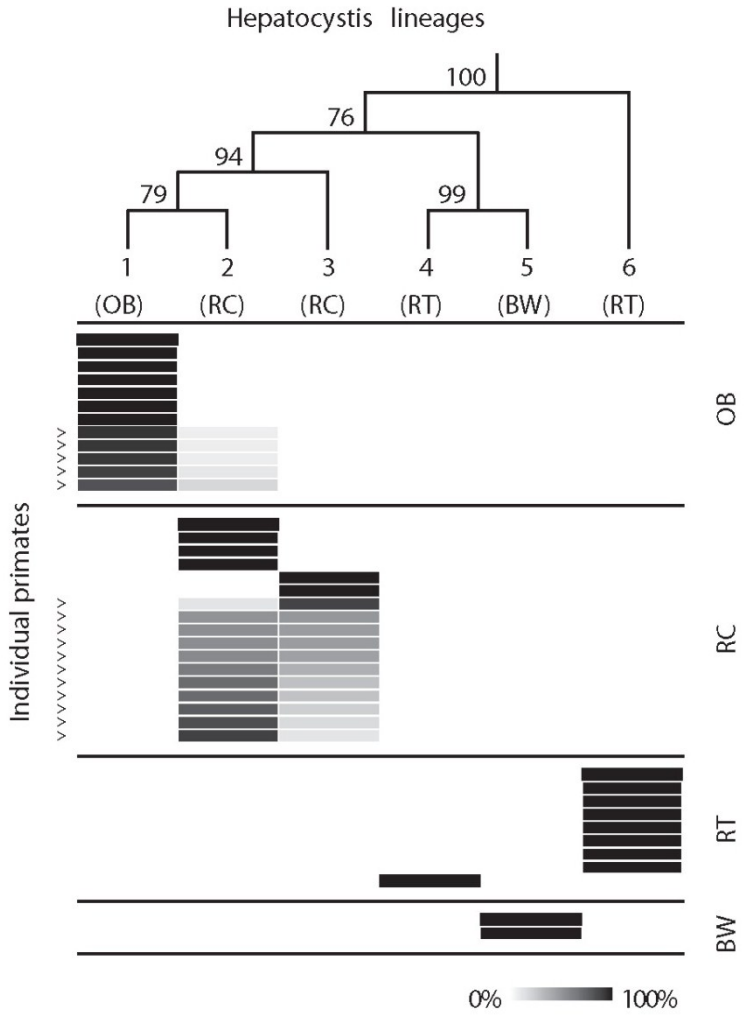

Figure 4.3: Mixed-lineage *Hepatocystis* co-infections in Kibale primates. Rows represent 40 primates subjected to "deep" pyrosequencing of a 420-bp amplicon of the parasite cytochrome b gene. Cells are shaded in proportion to the percent of individual high-quality pyrosequencing reads mapping to each parasite lineage. The cladogram on the top of the figure shows a simplified topology of the phylogeny shown in Figure 4.2A, with numbers representing bootstrap values. Host species abbreviations (BW = black-and-white colobus, OB = olive baboon; RC = red colobus; RT = red-tailed guenon) and lineage numbers (1-6) follow Figure 4.2. Thickened lines on the vertical axes indicate primates with mixed-lineage co-infections, as evidenced by pyrosequencing reads mapping to more than one lineage.

LINKING STATEMENT 3

In the previous chapter, I examined the host range and transmission of blood parasites within the Kibale primate community. Surprisingly, *Plasmodium* was not detected in any samples. Six lineages of *Hepatocystis* were discovered, which appeared to be extremely host species-specific. However, deep-sequencing revealed that 16 individuals out of 40 were co-infected with *Hepatocystis* lineages. Five of these 16 individuals were baboons that were co-infected with minor variants of a red colobus-specific lineage.

Transmission of *Hepatocystis* appears to be dominated by host factors. All primates in this community physically overlap to varying extents, and the fact that infections are not shared suggests physiological or immunological host barriers are preventing infection. Theory suggests that parasites with rapid generation times and exposure to multiple host species through their vectors would be multi-host parasites. However, my results suggest that these parasites are host specialists. The tendency of blood parasites towards host specialism indicates that extensive adaptation to specific hosts may be required. However, why baboons may be susceptible to red colobus-specific lineages of *Hepatocystis* remains an unknown, since these hosts do not typically interact, share food sources, or niche space. I speculate that vector feeding preference may be driving this relationship.

The host range of *Hepatocystis* greatly contrasts with those of *Oesophagostomum* and *Trichuris*. Specifically, blood parasite lineages show host specialization, while soil transmitted helminth lineages show (on average) far more generalism.

Plasmodium was not discovered in wild primates, and *Hepatocystis* was not discovered in the adjacent human population. Therefore, this research was divided into two parts. The next section, Chapter 4 Part II, examines the species of *Plasmodium* in humans, and takes advantage of my multi-method diagnostic procedure for identifying *Plasmodium* (intended to ensure that even unique, potentially zoonotic blood parasites emerging from the primate population were detected) to evaluate the problem of malaria over-diagnosis in this region.

CHAPTER 4: PART II

Multi-method assessment of patients with febrile illness reveals over-diagnosis of malaria in rural Uganda

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4.6 Abstract

4.6.1 Background

Health clinics in rural Africa are often resource limited. As a result, many or all patients presenting with fever are treated with antimalarials, even without diagnostic testing to confirm disease. Malaria rapid diagnostic tests (MRDTs) offer opportunity to clinically diagnose malaria quickly and inexpensively. This study sought to identify the utility and accuracy of MRDTs relative to a staple molecular diagnostic, polymerase chain reaction (PCR) and DNA sequencing, in a rural setting in Uganda where diagnoses of malaria are made based on symptoms alone.

4.6.2 Methods and Findings

Patients presenting with fever at one of two health clinics in the Kabarole Distrct of Uganda were enrolled in this study. Blood was collected by finger prick and used to administer MRDTs, make blood smears for microscopy, and blot Whatman FTA cards for DNA extraction, amplification, and sequencing. The accuracy of MRDTs and microscopy were assessed relative to PCR. A total of 71 patients were enrolled, and 31 were diagnosed with *Plasmodium* infection by at least one method. Percent parasitaemia, an estimate of the intensity of infection, was greatest in children under five years of age. Comparing diagnostic pairs determined that MRDTs and microscopy performed similarly, and both were slightly more likely to miss malaria-positive samples than to diagnose malaria in truly negative samples. When the two methods were combined, diagnoses were near-perfect. However, both missed one case of non-*P. falciparum* malaria (*P. malariae*) that was identified and characterized by PCR and sequencing. The majority of patients would have been misdiagnosed with malaria, had diagnosis been based on symptoms alone.

4.6.3 Conclusions

While malaria remains a predominant illness in this region, over-diagnosis of the disease is a serious concern. Our results suggest that all three methods are highly effective for diagnosing malaria. PCR and sequencing was the only method to detect a non-*P. falciparum* species, and gives the advantage of species-level identification, but is not accessible in most settings. Diagnostically, MRDTs are the same or better than microscopy for detecting malaria, but are more feasible in rural or resource-limited areas. Diagnosis of malaria by symptoms alone appears to be highly inaccurate in this setting.

4.7 Introduction

In Africa, 70% of fevers are initially managed at home, with traditional remedies and bed rest used to alleviate symptoms (Amexo *et al.* 2004). It is only when symptoms continue to worsen that medical attention may be sought, and even at this stage, patients may not receive proper diagnosis – especially at remote or rural health facilities that are often resource-limited. The symptoms of malaria overlap with a number of other illnesses, making accurate diagnosis difficult, even among experienced practitioners (Rooth & Björkman 1992; Olaleye *et al.* 1998; Kilian *et al.* 2000; Chandramohan *et al.* 2002). In addition, antimalarials can be prescribed by primary care workers who vary in experience and training. As a result, most patients with a recent history of fever are diagnosed with and treated for malaria, despite the fact that a number of other illnesses, including pneumonia, typhoid fever, respiratory tract infections, transient viral illnesses and meningitis may be causing clinical disease (Källander *et al.* 2004; Bell *et al.* 2006; Uneke 2008). Therefore, the sensitivity of malaria diagnosis at clinics may be as high as 100% since few patients with clinical malaria are missed, but the specificity is often extremely low (Oliver *et al.* 1991; Rooth & Björkman 1992; Chandramohan *et al.* 2002). Indeed, a study conducted in Tanzanian hospitals showed that less than half of all individuals symptomatically diagnosed for malaria were actually positive for the parasite by microscopic examination of blood smears (Reyburn *et al.* 2004). Individuals treated for malaria but not actually harboring the infection were often not prescribed antibiotics, and were more likely to die than individuals with malaria (Reyburn *et al.* 2004). These results suggest that the symptomatic or presumptive diagnosis of malaria is costly not only in terms of wasted antimalarial drugs, but also in the morbidity and mortality associated with misdiagnosis.

In many malaria endemic regions, safe, inexpensive antimalarials such as chloroquine have become ineffective due to the emergence of drug resistant parasites. These drugs are being replaced by more toxic and expensive alternatives, including sulfadoxine-pyramethamine (Redd *et al.* 1996). Prescribing antimalarials through parasite-based diagnosis rather than symptomatic diagnosis offers a way to appropriately dispense drugs, but can be challenging in locations with already overburdened health infrastructure. Specifically, the gold standard of malaria diagnosis is still considered microscopic examination of peripheral blood smears. However, this requires good quality reagents, clean equipment, functioning microscopes, workspace, and skilled

personnel (Bell *et al.* 2006). To circumvent poor diagnostic access, antigen-based malaria rapid diagnostic tests (MRDTs) have been implemented with success in field conditions, especially in regions without prior access to microscopic diagnostics (Ansah *et al.* 2010; Masanja *et al.* 2010; Wilson 2012).

This study sought to identify the frequency of malaria misdiagnosis in two health clinics in Uganda, where transmission of this parasite is stable across 95% of the country (Okello *et al.* 2006). Despite a global decrease in malaria since 2000, Uganda is one of only four East African countries where both documented cases of malaria and deaths have increased; in Uganda's case by nearly 50% (W.H.O. 2011). Furthermore, Uganda's malaria diagnosis recommendations still include presumptive diagnosis of malaria in patients with fever or a recent history of fever (Republic-of-Uganda 2005). This is despite World Health Organization (WHO) guidelines that suggest using presumptive diagnosis only when clinical diagnostic methods are unavailable (W.H.O. 2010). Using three methods (traditional microscopic examination of blood smears, MRDTs, and polymerase chain reaction (PCR)), we explore the rate of misdiagnosis in a rural community in the Kabarole District of Uganda that lacks regular access to malaria diagnostics.

4.8 Methods

4.8.1 Blood Sample Collection and Processing

Two rural health clinics located within the Kabarole District of Uganda were enlisted in this study. Patients visiting these clinics in June and July 2011 with a fever (an axillary temperature of 37.5°C or higher), who were not pregnant, and who had not treated their symptoms with standard antimalarials were asked to participate. Following informed consent, blood samples were collected by finger prick. One drop of blood was used (as per manufacturer instructions) in a Nova Century Scientific Rapid Diagnostic Test for HRP-2 protein of *Plasmodium falciparum* – the predominant species of malaria parasite in the region. An additional two drops of blood were used to make thick and thin blood smears for microscopic diagnosis. Finally, one to five additional drops were collected on Whatman FTA Classic Cards for PCR. All patients were offered complimentary treatment based on MRDT results and the health practitioner's diagnosis. Blood samples were shipped to North America for microscopic examination and molecular diagnostics.

4.8.3 Microscopy

Thin and thick smears were stained with Geimsa and viewed under 100X oil immersion objective magnification. Thick smears were used to confirm the presence of blood parasites, while thin smears were used to confirm speciation and determine infection intensity (percentage parasitaemia). For each thin smear, a field containing a representative monolayer of red blood cells (RBCs) was identified and all RBCs in that field were counted. This was repeated five times to yield an average number of RBCs per field. One hundred fields, or the maximum number of viewable fields on the slide, were scanned for intra-erythrocytic parasites morphologically consistent with *Plasmodium*. Intensity of infection in positive smears was calculated following a previously described formula (Thurber *et al.* 2013).

4.8.4 DNA Extraction, PCR, and Sanger Sequencing

DNA was extracted from Whatman FTA Classic Cards following modified manufacturer's instructions. Briefly, a four millimeter sample disc was punched from each FTA card and placed in a PCR amplification tube. FTA purification reagent (200 µl) was added to each tube and incubated for five minutes at room temperature. The FTA purification agent was removed by pipette, and this process repeated an additional three times. 200µl of TE buffer (10mM Tris-HCl, 0.1 mM EDTA, pH 8.0) was then added to each PCR tube and incubated for five minutes at room temperature. TE buffer was removed by pipette and this process was repeated once more. The sample disc was allowed to dry completely at room temperature before being subjected to PCR amplification.

A semi-nested PCR targeting the cytochrome b (cytb) gene of *Plasmodium* was conducted using primers CytB3384F (5'-GTAATGCCTAGACGTATTCCTG-3') and CytB4595R (5'-GTTTGCTTGGGAGCTGTAATC-3') in the external reaction, and CytB3706F (5'-GTTTGCTTGGGAGCTGTAATC-3') and CytB4595R in the internal reaction. This procedure generated amplicons of 1254 bp (external) and 932 bp (internal) predicted size (Thurber *et al.* 2013). Both external and internal reactions were performed in 25 µl volumes using the FailSafe system (EpiCenter Biotechnologies, Madison, WI, USA), with reactions containing 1X FailSafe PCR PreMix with Buffer E, 1 Unit of FailSafe Enzyme Mix, 2.5 picomoles of each primer, and 1 µl of template. Reactions were cycled using previously described profiles (Thurber *et al.* 2013).

For samples identified as positive for *Plasmodium* by PCR but negative by microscopy or MRDT, amplicons were Sanger sequenced in both directions using internal primers CytB3706F and CytB4595R on ABI 3730xl DNA Analyzers (Applied Biosystems, Carlsbad, CA) at the University of Wisconsin Biotechnology Center DNA Sequence Facility. Sequences were hand-edited using Sequencher v4.9 (Gene Codes Corporation, Ann Arbor, MI). To identify parasite species, newly generated DNA sequences were compared to reference sequences in Genbank using BLASTn, a tool from the National Center for Biotechnology Information (NCBI).

4.9 Results and Discussion

A total of 78 patients were enlisted in this study; 38 women and 40 men. All patients were tested by MRDT. Blood samples for PCR were collected from 71 of these patients, and blood samples for microscopy from 67 patients. Parasitaemia of *Plasmodium* positive blood slides ranged from 0.003% to 3.399% of total RBCs. Mean parasitaemia of children aged 5 or younger was significantly higher than in patients older than 5 years (mean of 18 patients $\leq$5 years = 1.26%, mean of 60 patients >5 years = 0.1585%; Wilcoxon rank sum test, W = 117, $p = 0.005$).

We used PCR as the primary reference standard because it is established to outperform microscopy in sensitivity and specificity (Humar *et al.* 1997; Ohrt *et al.* 2002; Farcas *et al.* 2003). PCR identified 27 patients as positive for *Plasmodium*. Microscopic examination of peripheral blood smears identified 28 patients as positive for *P. falciparum*, and MRDTs identified 32 patients as positive. Comparing the sensitivity and specificity of diagnostic methods identified that MRDTs and microscopy performed similarly; both were over 90% sensitive and specific, although MRDTs were marginally better at detecting infection when it was present (sensitivity) and returning negative results when the parasite was truly absent (specificity; Table 1). Both tests had higher specificity than sensitivity, suggesting that both are slightly more likely to miss malaria than to over-diagnose it (Table 1). Parallel testing was also conducted, where the results of MRDTs and microscopy were combined. These methods together are 96% sensitive and 100% specific, suggesting that malaria-positive patients may occasionally be missed, but malaria-negative patients would never be misdiagnosed as positive. It should be noted that two patients were negative by PCR but positive by both other methods. Other studies have suggested that false positives can be the result of recently cured malaria (Farcas *et al.* 2003). However,

given that parasites were detected microscopy in addition to MRDT makes it possible that instead, PCR failed to detect parasites from these samples. This would suggest that field of malaria diagnostics still lacks an infallible gold standard.

MRDTs and microscopy combined were only 96% sensitive because both missed one patient sample that was positive by PCR. Sequencing revealed that this sample shared 100% sequence identity with two published *P. malariae* cytochrome b sequences (Genbank Accession Numbers AB489194 (unpublished) and AB354570 (Hayakawa *et al.* 2008); BLASTn). Another sample was positive by PCR and microscopy (parasitaemia = 0.013%) but negative by MRDT. Sequencing identified this sample as 99% identical to sequences from a published set of *P. falciparum* cytochrome b sequences from India (Tyagi *et al.* 2014). Finally, one sample was positive by PCR and MRDT but negative by microscopy. This sample was also determined to be *P. falciparum*, sharing 100% identity with sequences from the aforementioned Indian population set (Tyagi *et al.* 2014). These results suggest that both MRDT and microscopy can miss *P. falciparum* infection, but that the probability of missing other malaria species, like *P. malariae,* is even greater. In our study, MRDTs specific for *P. falciparum* were selected based on this species' ubiquity in the study area. However, these results highlight the limitations of species-specific MRDTs, especially the potential to misdiagnose malaria infection based on a false-negative result from another parasite species.

All individuals in this study were symptomatically diagnosed with malaria by the presence of fever. However, our results suggest that only 43.59% were truly infected with *Plasmodium.* Thus, the majority of patients (56.41%) were misdiagnosed and would have been treated with antimalarials if not for diagnostic intervention. These results corroborate findings of malaria over-diagnosis elsewhere. For example, a cross-sectional study evaluating Uganda's policy of treating febrile illness with antimalarials reported rates of over-diagnosis ranging from 45.3% to 80.9% (Nankabirwa *et al.* 2009). This is a widespread phenomenon occurring throughout malaria endemic regions, with overestimates of malaria typically being greater than 30% (Amexo *et al.* 2004). Complex motivations of healthcare practitioners may account for malaria over-diagnosis –including peer or patient pressure, desire to prevent childhood mortality, and personal biases (Chandler *et al.* 2008). However, recent research has shown that simple interventions, including

training local medical personnel on the expected prevalence of malaria in their region and the issues surrounding over-diagnosis can significantly increase the accuracy of malaria diagnosis (Allen *et al.* 2013).

The new generation of MRDTs satisfy all criteria required for implementation in rural settings, being easy to use, inexpensive, and durable over the long-term in tropical conditions (D'Acremont *et al.* 2009). Our study adds to information on MRDT efficacy, showing that these tests were highly sensitive and specific, and were the only method capable of diagnostically testing all patients without missed samples. Indeed, in our study, MRDTs slightly outperform the traditional gold standard, microscopy, which is consistent with the findings of others (Ochola *et al.* 2006). However, only PCR detected non-falciparum parasites, and is the only method that offers species-level diagnoses. Nevertheless, the cost and expertise required for PCR render it impractical in the majority of settings. On the other hand, the ease and rapidity of MRDTs makes this method a promising approach for reducing malaria over-diagnosis, even in resource-limited settings. This confirms that MRDTs offer a promising approach to alleviate the costs of presumptive diagnosis in Africa's high and medium-high transmission regions.

Table 4.2: Sensitivity and specificity of MRDTs and microscopy relative to PCR. The diagnostic efficiency of three methods was assessed through sensitivity (*i.e.*, the probability of tests correctly identifying malaria presence) and specificity (*i.e.*, the probability of tests correctly identifying malaria absence). True readings indicate agreement between both diagnostic methods. False readings indicate discrepancies between diagnostic methods. The gold standard of each diagnostic pairing was considered to be polymerase chain reaction (PCR). Therefore, false positive readings are discrepancies where PCR returned a negative result while the non-gold standard returned a positive; false negatives are the opposite.

Diagnostic Comparison	True +	True -	False +	False -	Sensitivity (95% CI)	Specificity (95% CI)
PCR vs. MRDT	25	42	2	2	92.6 (75.6 – 98.9)	95.5 (84.5 - 99.3)
PCR vs. Microscopy	23	34	2	2	92.0 (73.9 - 98.8)	94.4 (81.3 -99.2)
PCR vs. MRDT + Microscopy	24	34	0	1	96.0 (79.6 – 99.3)	100 (89.6-100)

LINKING STATEMENT 4

In the preceding three chapters, I examined the host range and transmission of two groups of parasites: soil transmitted helminths (*Oesophagostomum*, Chapter 2; *Trichuris*, Chapter 3) and blood parasites (*Hepatocystis,* Chapter 4 Part I; *Plasmodium*, Chapter 4 Part II). Despite being transmitted within the same primate community, the transmission dynamics of these two groups starkly contrast with each other. While lineages of both soil transmitted helminths varied in host range, both *Oesophagostomum* and *Trichuris* contained one or more lineages that were broadly transmissible among wild primates and the adjacent human population. In contrast, *Plasmodium* was only discovered in humans, and each wild primate species (that was positive for blood parasites) had their own host-specific *Hepatocystis* lineage. Although the sample size is limited to four parasites in this thesis, my results suggest clear differentiation in host range between these parasite groups that is driven by the parasite's mode of transmission and life history traits, and not the availability or structure of the host community.

With the exception of *Plasmodium*, the parasites examined in this thesis do not typically result in death for the host. Therefore, my final data chapter explored the possibility that even non-lethal parasites can impose measurable consequences on their hosts. To examine this possibility, I used the focal parasite of Chapter 3, the whipworm *Trichuris*. I chose this parasite based on the results of Chapter 3, which confirmed that *Trichuris* was common, and that microscopy accurately diagnosed whipworm infection (as compared to PCR). As a host, I used the endangered red colobus monkey, which has been the focus of long-term study for over a decade.

CHAPTER 5

Sickness behaviour associated with non-lethal infections in wild primates

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5.1 Abstract

5.1.1 Background

Parasites that directly result in host mortality have received considerable attention. However, the majority of parasites in wildlife do not typically kill their hosts. These parasites, like helminths, may still cause reductions in host fitness or reproduction, but fitness costs are difficult to measure. Changes in host behaviour that result from infection can be measured as proxy for fitness effects. Behavioural changes in response to infection are termed "sickness behaviours", which often manifest as increased lethargy, somnolence, and reduced appetite. Here, we investigate the potential effects of a non-lethal helminth on the behaviour of a wild primate host.

5.1.2 Methods and Findings

We paired infection data from a ubiquitous soil transmitted helminth, the whipworm (genus *Trichuris*) with activity data from a habituated group of red colobus monkeys (*Procolobus rufomitratus tephrosceles*) in Kibale National Park, Uganda. We use generalized linear mixed models to examine the relationship between non-lethal parasitism and red colobus behaviour. Our results indicate that red colobus lessened energetic behaviour, but increased resting when positive for whipworm eggs in feces. Temporal patterns of behaviour also changed, with individuals switching behaviour less frequently when whipworm-positive. Feeding frequency did not differ, but red colobus consumption of plants from the legume family, Fabaceae, which are used locally in traditional medicines, was nearly twice as high in animals shedding whipworm eggs, as was bark consumption. These results suggest self-medicative plant use, although additional work is needed to verify this conclusion.

5.1.3 Conclusions

Our results match characterizations of sickness behaviours, which are considered an adaptive response elicited by hosts during infection. Induction of sickness behaviour, in turn, suggests that these primates are clinically sensitive to non-lethal parasite infections.

5.2 Introduction

Monitoring the health and abundance of wildlife is a primary focus of conservation, and parasitic diseases are increasingly being recognized as a major conservation concern (Deem *et al.* 2001; Harvell *et al.* 2002; Thompson *et al.* 2010). However, while parasites that cause direct host mortality receive considerable research attention, the majority are non-lethal under ordinary circumstances. Often, non-lethal parasitic infections are caused by macroparasites (e.g., gastrointestinal helminths) that may not produce overt clinical symptoms, but are instead responsible for chronic, low-intensity infections (Gulland 1995; Hudson & Dobson 1995). These infections may impose long-term consequences, including reductions in host fitness and reproduction (Anderson 1978; Anderson & May 1979).

Defining and measuring traits that predict fitness is difficult, especially in wildlife populations where intensive, long-term field monitoring is necessary (Barker 2009). However, considerable research has documented changes in host behaviour resulting from parasitism (Minchella & Scott 1991; Poulin 1994). Parasite-induced behavioural changes can be complex (as in *Toxoplasma,* for example (Berdoy *et al.* 2000)), but most are subtle and involve reductions in host activity levels, which often manifest as lethargy, somnolence and reduced appetite (Hart 1988). Previously, these "sickness behaviours" were considered maladaptive – a consequence of the parasite exerting pathological effects on the host (Poulin 1995). Increasingly, however, sickness behaviours are being reinterpreted as adaptive changes induced by the host to conserve energy and fight infection (Hart 1988; Poulin 1995; Johnson 2002).

Despite a basic understanding of the immunological underpinnings of sickness behaviour (Dantzer 2001), little research has attempted to describe the presentation of sickness behaviours in wild animal populations (Krief *et al.* 2005b; Weary *et al.* 2009). Here, we examine behavioural variation in a wild primate associated with a gastrointestinal nematode, the whipworm (genus *Trichuris*). Whipworms are globally ubiquitous, soil-transmitted helminths of increasing concern to public health (Stephenson *et al.* 2000; Bethony *et al.* 2006). In humans, children aged 5-15 years often suffer the most pronounced symptoms, ranging from gastrointestinal upset to cognitive and developmental stunting. In severe infections, Trichuris Dysentery Syndrome may result in rectal prolapse and chronic dysentery (Stephenson *et al.*

2000; Bethony *et al.* 2006; Ojha *et al.* 2014). Recently, ribosomal DNA sequencing revealed nearly identical sequences between a number of Ugandan primates (including monkeys and chimpanzees) and humans, suggesting that some parasite lineages are broadly transmissible among primates, including humans (Ghai *et al.* In Press). This is especially concerning given that whipworms are commonly found in African primates that share environments with humans (Murray *et al.* 2000; Gillespie *et al.* 2005b; Mbora & Munene 2006; Weyher *et al.* 2006; Teichroeb *et al.* 2009; Ghai *et al.* In Press).

We focus on wild red colobus monkeys (*Procolobus rufomitratus* ssp. *tephrosceles*), which are arboreal, predominantly leaf-eating primates. Currently, this taxon is considered endangered, with the largest and potentially only viable population existing in the location of our study, Kibale National Park, Uganda (Struhsaker 2005). Using activity data from a habituated group, we quantify changes in: 1) activity, 2) rate of behaviour change, and 3) feeding pattern associated with whipworm infection.

5.3 Methods

5.3.1 Study Site and Data Collection

This research was conducted in Kibale National Park (hereafter Kibale), a 795 km^2 tropical rainforest in southwestern Uganda (Chapman & Lambert 2000; Chapman *et al.* 2010). In the Kanyawara region, one habituated group of over 100 red colobus is the focus of long-term research and was used in this study (Gogarten *et al.* 2014). Over 48 months from 2007 to 2011, we collected activity data from individuals that were recognisable based on physical characteristics and collars affixed as part of a larger project. Activity data were collected in the morning and early afternoon by selecting five easily visible adults every 30 minutes and recording their identity, sex, and behaviour at the moment of observation. If the animal was feeding, the plant species and part being eaten were also recorded.

Fecal samples were collected opportunistically when animals were observed defecating. In a field laboratory, these samples were subjected to a modified ethyl acetate sedimentation method using one gram of feces to concentrate nematode eggs, which are shed in the feces of infected individuals (Young *et al.* 1979; Garcia *et al.* 2005; Greiner & McIntosh 2009; Ghai *et al.* In

Press). Sedimented samples were preserved in formalin and scanned under 10X objective light magnification to determine whipworm infection status. Samples were classified as "positive" when whipworm eggs were detected and "negative" when the entire sediment was scanned without discovering eggs. While positive samples are conclusively infected with whipworm, negative samples may represent uninfected individuals, or infected individuals that are not actively shedding eggs. The number of eggs per gram of feces (EPG) was also recorded, as this is considered a proxy for adult nematode infection intensity in some species (Cabaret *et al.* 1998; Seivwright *et al.* 2004).

Finally, to determine whether the temporal pattern of red colobus behaviour varied with infection, three months of focal animal data were collected from 16 well-known adults of the same red colobus group from June – September, 2013 inclusively (eight females, eight males). Focal animals were observed in 30 minute sampling periods, during which all activity and behaviour were recorded. A corresponding fecal sample was collected the same day and processed as described above.

5.3.2 Analysis

All statistical analyses were conducted in R (R-Development-Team 2008) unless otherwise specified. For each fecal sample, one week of behavioural data (three days before the date of fecal sample collection and four days after) from the corresponding individual was examined. We used the activity data only for the week from which the sample was collected based on known variability in fecal egg detection from human infections (Hall 1981). Only samples from individually recognisable animals, and individuals whose infection status changed through time (to control for intra-individual variation in behaviour) were included. Prior to performing further analysis, the relationship between whipworm infection (prevalence and EPG) and season (average rainfall and temperature in the Kanyawara region of Kibale) were examined using linear regression. There was no discernible relationship (results not shown), and therefore any potential seasonal effects of infection were considered negligible for this parasite.

Red colobus behaviour was categorized into eleven activities (breastfeed, chase, copulate, feed, fight, groom, move, play, present, rest, vocalize). However, six behaviours did not occur or were

exceedingly rare (<1% of all observations; breastfeed, chase, fight, play, present, vocalize), and were therefore omitted from analyses. The five remaining behaviours (copulate, feed, groom, move, and rest) were converted into proportions reflecting the number of events for that behaviour relative to the total number of observations. The relationship between whipworm infection and red colobus activity was evaluated using generalized linear mixed effects models (GLMMs). Separate GLMMs were constructed for each behaviour, with behaviour a binomial response variable (*e.g.* feed/not feeding). Models containing infection as a binary variable (either positive or negative for whipworm eggs in feces) outperformed those that used EPG. Furthermore, the very low egg counts in all samples (<100 EPG) precluded binning the data into several discrete categories, like uninfected, low EPG, medium EPG, and high EPG. Therefore, final GLMMs included individual identity as a random intercept, and contained two fixed effects: (i) sex and (ii) binary infection status. GLMMs were fitted with a binomial error distribution and implemented using the *lme4* library . Statistical significance of fixed effects was assessed using likelihood ratio tests. We also calculated change in the relative frequency of behaviour (to account for differences in the occurrence of each behaviour) using the following equation:

$$((pi_{[infected]} / pi_{[uninfected]}) - 1) * 100$$

Here, pi indicates the number of observations during which a given behaviour was observed out of the total number of observations per individual.

The relationship between all five behaviours was visualized by principal component analysis (PCA). Proportional mean activity per individual at whipworm-positive and negative time points were transformed into individual scores for the first two components and plotted following standardization around zero on each axis. Infection status was color coded and the minimum area of a given status was outlined using convex hulls. Each individual was represented twice in this analysis; once at whipworm-positive intervals and again at whipworm-negative intervals. PCA was used to visualize all activities together – the aforementioned analyses were performed with GLMMs as describe above.

Rate of behavioural switching was estimated from focal sample data, to determine if presence of whipworm affected temporal patterns of behaviour. To control for intra-individual variation in behaviour, only individuals for which infection status changed during the three month focal period were included, resulting in a final sample size of 14 individuals. For each focal individual, we calculated the mean time (in seconds) per behaviour (breastfeed, chase, copulate, feed, fight, give groom, receive groom, move/travel, play, and rest) over a 30 minute focal period. The mean time an animal spent performing a behaviour was averaged within focal individuals at whipworm-positive and negative intervals and the probability of behaviour switching was evaluated using the Kaplan-Meier method (Bland & Altman 1998). Log-rank tests were used to assess whether there were significant difference between the curves of whipworm-positive and negative individuals using the *survival* library (Therneau 2000).

Finally, to examine the relationship between feeding and infection, the proportion of time red colobus spent feeding on a particular plant part (bark, flower, fruit, leaves, seeds, or other [leaf petioles, leaf buds, pith, dead wood, and soil]) was compared between whipworm-positive and negative intervals using binomial tests. A Bonferonni correction was applied to critical *p*-values to account for multiple comparisons. Since plants evolve compounds with medicinal benefits, we also explored the taxonomic composition of plant families consumed at whipworm-positive and negative intervals using the net relatedness index to quantify the phylogenetic structure of plants in the diet (Webb 2000) (Appendix B.1, Figure B2).

5.4 Results

In total, 371 fecal samples and 3032 individual activity recordings were included from 43 individuals (28 female, 15 male; Table B1). Of these, 155 (41.8%) fecal samples were positive for whipworm eggs. Males were more likely to have whipworm-positive samples (56%) than females (35%; $\chi^2 = 13.46$, $p < 0.001$). Mean whipworm EPG was 12.69 (±16.08 S.D.), and this also varied by sex, with males having higher EPG (17.5 EPG ±8.08 S.D.) than females (7.85 EPG ±8.82 S.D.; Wilcoxon rank sum test $p = 0.01$).

GLMMs that included both sex and binary whipworm infection status had highest support for all behaviours except feeding (Table 5.1). GLMMs revealed clear sex differences, with males

copulating and resting more, and moving and grooming less (Table 5.1, Figure B1). There was no difference in the proportion of time spent feeding between sexes. In both sexes, behaviour varied with whipworm status.

Whipworm-positive individuals copulated, groomed and moved less and rested more (Table 5.1, Figure B1). As with sex, feeding did not vary with infection status. Relative time spent copulating differed most dramatically with infection status, being 71.72% (±4.2 S.E.) lower in females and 56.03% (±11.6) lower in males when whipworm-positive (Figure 5.1). Feeding (no significant difference) and moving (27.01% lower in females and 25.84% lower in males when whipworm-positive) changed the least with infection. Resting was the only behaviour that increased significantly in whipworm-positive individuals, being 34.10% (±2.8 S.E) higher in females and 23.64% (±6.2 S.E.) higher in males (Figure 5.1).

Principal component (PC) 1 and 2 explained 65.59% and 21.41% of the total variance, respectively. Individuals occupied more negative values in PC 1 and more positive values in PC 2 when positive for whipworm (Figure 5.2).

Focal animal data from 14 red colobus revealed that individuals switched behaviours less often when whipworm-positive than when whipworm-negative ($\chi^2 = 11.96$, $p < 0.001$). The average time to a switch in behaviour was 70.45 seconds (median = 63.5 sec) when individuals were whipworm-negative, and 222.08 sec (median = 204.78 sec) when individuals were whipworm-positive (Figure 5.3).

While the proportion of time spent feeding on most plant parts (flowers, fruit, leaves, seeds, other) was the same regardless of infection status, the proportion of time spent eating bark increased ($p = 0.012$ from binomial tests; Table 5.2) when individuals were whipworm-positive (whipworm-negative = 3.84%, whipworm-positive = 7.39%). The taxonomic composition of plants consumed by red colobus tended to be clustered, irrespective of infection status (whipworm-negative: NRI = 3.02, p = 0.001; whipworm-positive: NRI = 3.64, p = 0.001). However, red colobus feeding frequency differed with infection status for one particular plant family, Fabaceae (whipworm-negative: 23.28%; whipworm-positive: 31.58%, Bonferroni

corrected $\chi^2 = 12.87$, $p < 0.05$). Specifically, this difference resulted from increased consumption of a Fabaceae genus with known medicinal properties, *Albizia*, from 23.6% of all Fabaceae plant choices to 45.0% in whipworm-positive individuals ($\chi^2 = 14.31$, $p < 0.001$; Table B2).

5.5 Discussion

We investigated the relationship between host behaviour and infection with a non-lethal gastrointestinal parasite, the whipworm, *Trichuris* spp. Individual behavioural profiles differed considerably with infectious status (Figure B1). Some of this variation may be the result of differences in infection intensity, fitness or immunity between individuals, co-infections, or social circumstance (Schmid-Hempel 2003; Lopes 2014). However, our results suggest that sex differences may also be important, since males had more positive samples and greater egg burdens than females.

Regardless of sex, red colobus rested more and moved less, groomed less, and copulated less when whipworm-positive. These patterns may indicate that that red colobus modify their behaviour because of parasitism. However, it is also possible that red colobus may alter certain behaviours for reasons other than whipworm infection, and these factors may also affect whipworm egg shedding. For example, an increase in stress (perhaps from injury or antagonism) may cause increased resting behaviour but is also immunosuppressive, potentially facilitating parasite infection or the release of eggs. Another possibility is that red colobus with affinity for "risky" behaviours are more likely to become infected with whipworm. For example, individuals that frequently groom conspecifics could be more susceptible to ingesting larvated parasite eggs that catch on the fur (Nunn & Altizer 2006). Similarly, animals that move often might come into more frequent contact with infective stages that contaminate the soil and vegetation (Nunn & Altizer 2006). However, our results are contrary to those expected if behaviour were causing infection – animals rested more when whipworm-positive. Our results are more consistent with sickness behaviours, which imply that infection causes changes in behaviour. Further, our results suggest an energetic trade-off, whereby "sick" individuals favor low energy states over strenuous activity. Specifically, we found that the most energetically expensive behaviours, copulating and grooming, showed the greatest reduction during infection (see (Coelho *et al.* 1976) for estimates of energetic costs).

Anecdotes of lethargy during illness have been reported in chimpanzees. For example, a report from Kibale described increased resting and decreased feeding in animals experiencing a flu-like illnesses (Krief *et al.* 2005b). In a Tanzanian population, a sick female rested more and fed less than other group members, and constructed sleeping nests during the day (Huffman & Seifu 1989). Such behaviour could result directly from physiological impairment caused by the parasite. If so, it would imply a significant fitness cost of whipworm infection. However, an expanding body of evidence suggests that sickness behaviour has adaptive benefits to the host (Hart 1988; Johnson 2002). For example, early studies showed that rats repeatedly chose inactivity over exercise when injected with endotoxin (known to stimulate the immune response), suggesting for the first time that they were motivated to rest (Miller 1964). If sickness behaviour is an adaptive host response, then the behaviours observed in red colobus in our study could help maintain homeostasis during infection.

We did not find evidence that feeding decreased with infection, which contrasts with previous studies (Crompton 1984). However, we examined the frequency of feeding and not the rate of food intake. Another metric, such as bite rate, may have uncovered a different relationship. Nevertheless, we showed that whipworm-positive individuals consumed more bark. Bark is highly fibrous, has low nutritional value, and is relatively indigestible (Huffman 1997). Although it is a consistent component of many primate diets, very little is known about why it is eaten or its contribution to health. The bark from some plant species may have compounds with medicinal properties. For example, bark from *Gongronema latifolium* is eaten by the Bossou chimpanzees in West Africa, and is also used by local people as a purge for symptoms associated with intestinal parasites (Huffman *et al.* 2013). In addition, the highly fibrous nature of bark may increase gut motility, which would assist in purging intestinal nematodes (Huffman *et al.* 1996). We therefore speculate that increased consumption of bark might indicate self-medication in whipworm-positive individuals.

Additional evidence supporting self-medication in whipworm-positive red colobus comes from shifts in dietary taxonomic composition. Although we did not find differences in the phylogenetic structure of plants consumed, we observed an 8.3% increase in consumption of

plants in the legume family, Fabaceae (*a.k.a.* Leguminosae), in whipworm-positive individuals. In particular, consumption of two common species, *Albizia grandibracteata*, and *Albizia gummifera,* nearly doubled when animals were whipworm-positive. These species are used by local people as anti-parasitics (Burkill 1985; Krief *et al.* 2005a; Orwa *et al.* 2009), and *Albizia grandibracteata* contains saponins, which are known to have *in vitro* anti-helminthic activity (Krief *et al.* 2005a). Self-medication is considered a "truly" adaptive response to parasitic infection because it is complex, has convergently evolved in a number of hosts, and is beneficial to host fitness (Poulin 1995). However, in our study it was not possible to control for additional factors, such as seasonal patterns in plant availability, and our results remain correlative.

Finally, we found evidence that red colobus behavioural flexibility changed with whipworm status. Specifically, individuals took longer to switch behaviours when whipworm-positive. Reductions in the complexity of behavioural patterns have been associated with stressful conditions in a number of studies, and have also been used as a non-invasive indicator of health impairment (Alados *et al.* 1996; Maria *et al.* 2004). In primates, fractal analysis has been used to show that health impairment by intestinal parasites results in increased periodicity of behaviour (*i.e.*, reduced complexity) (Alados & Huffman 2000; MacIntosh *et al.* 2011). Complex or unpredictable behaviour is believed to be advantageous because it allows organisms to adapt to changing environments (Goldberger 1996; MacIntosh *et al.* 2011). Thus, while beneficial in the short term for mitigating the costs of infection, prolonged expression of sickness behaviours could have fitness costs.

In conclusion, we provide evidence that red colobus behaviour covaries with the presence of whipworm eggs in feces, and that this relationship results from the induction of sickness behaviours. Specifically, we document that shedding of eggs in feces was associated with more resting but less moving, grooming and copulating. In addition, red colobus ate plants with potential medicinal properties more frequently when shedding whipworm eggs. Sickness behaviours may help infected animals cope with the burden of infection by concentrating limited energetic resources on critical immune functions or by impairing the parasite. However, although such behaviours may be adaptive in the short term, they may have longer term fitness consequences. More generally, our results support the idea that non-lethal parasite infections can

have significant impacts on host behaviour. How these changes in behaviour may translate into fitness consequences remains to be determined, but offers a potential avenue for future research in the field of wildlife parasitology.

Table 5.1: GLMM results. Results of five independent generalized linear mixed models on the activity (proportion of events spent copulating, grooming, feeding, moving, or resting) of focal group red colobus monkeys (*Procolobus rufomitratus*). Models included the individual's sex and infection status (fecal samples were microscopically examined and classified as "positive" or "negative" based on the presence or absence of whipworm eggs of the genus *Trichuris*) as fixed effects, and the individuals themselves as random effects. Statistical significance (bolded values) of fixed effects was tested using likelihood ratio tests comparing the fit between a full model and a reduced model missing a given fixed effect.

Dependent Variable:	Independent Variables: Infection, Sex				Likelihood Ratio Test			
	Females		Males		Infection		Sex	
	Negative	Positive	Negative	Positive	χ^2	*p*	χ^2	*p*
Copulating	0.0198	0.0050	0.0443	0.0114	21.78	<**0.0001**	4.69	**0.0310**
Grooming	0.1032	0.0518	0.0563	0.0275	22.63	<**0.0001**	11.48	**0.0006**
Feeding	0.4774	0.4981	0.4732	0.4939	1.15	0.28119	0.02	0.8656
Moving	0.0934	0.0533	0.0665	0.0375	15.02	**0.0001**	4.02	**0.0345**
Resting	0.2806	0.3587	0.3350	0.4194	21.20	<**0.0001**	7.72	**0.0023**

Table 5.2: **Percentage of plant parts eaten by red colobus at whipworm-positive and negative intervals.** Non-parametric comparisons of two proportions were calculated using a Chi-squared test with Yate's continuity correction. *P*-values shown (bolded when significant) are adjusted for multiple comparisons using the Bonferroni correction method.

Part	% Negative (N=886)	% Positive (N=731)	χ^2	*p*-value
Bark	3.84	7.39	9.130	**0.012**
Flower	1.58	0.82	1.320	>0.500
Fruit	1.35	1.64	0.072	>0.500
Leaves	91.99	88.37	5.595	0.108
Seeds	0.11	1.09	5.311	0.126
Other	1.13	0.68	0.446	>0.500

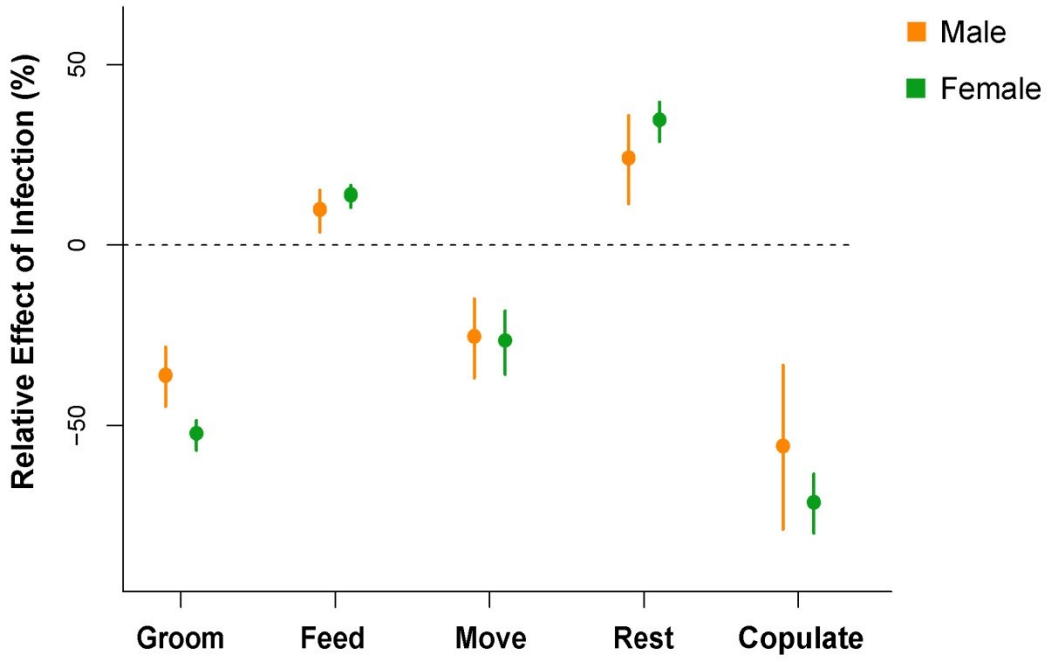

Figure 5.1: Mean relative change in each behavior as a function of infection. Relative change in the frequency of each behaviour was calculated for each sex (males = orange, females = green) such that negative values indicate a reduction in the frequency of a behavior when individuals were whipworm-positive. Error bars = s.e.m.

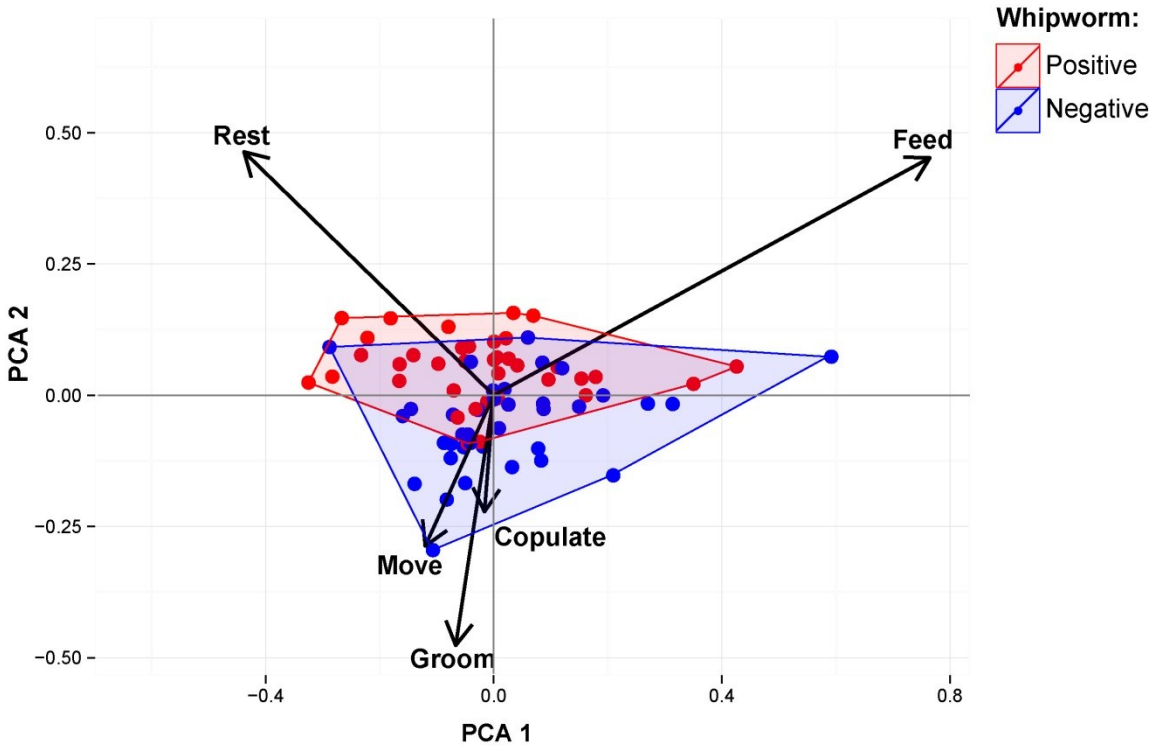

Figure 5.2: **Principal Component Analysis visualization of all five behaviours.** First two principal components (PC 1 and PC 2) shown (cumulative proportion of explained variance = 86.9%). PC 1 loadings were: Feed = 0.788, Rest = -0.601, and Move = -0.127. PC 2 loadings were: Feed = 0.361, Rest = 0.630, Move = -0.634, and Groom = -0.257. Data points represent the mean variation in behaviour of an individual at time points when negative for whipworm eggs (blue) or positive for whipworm eggs (red) in feces, and convex hulls occupied by individuals when negative (blue) or positive (red) outlined and shaded.

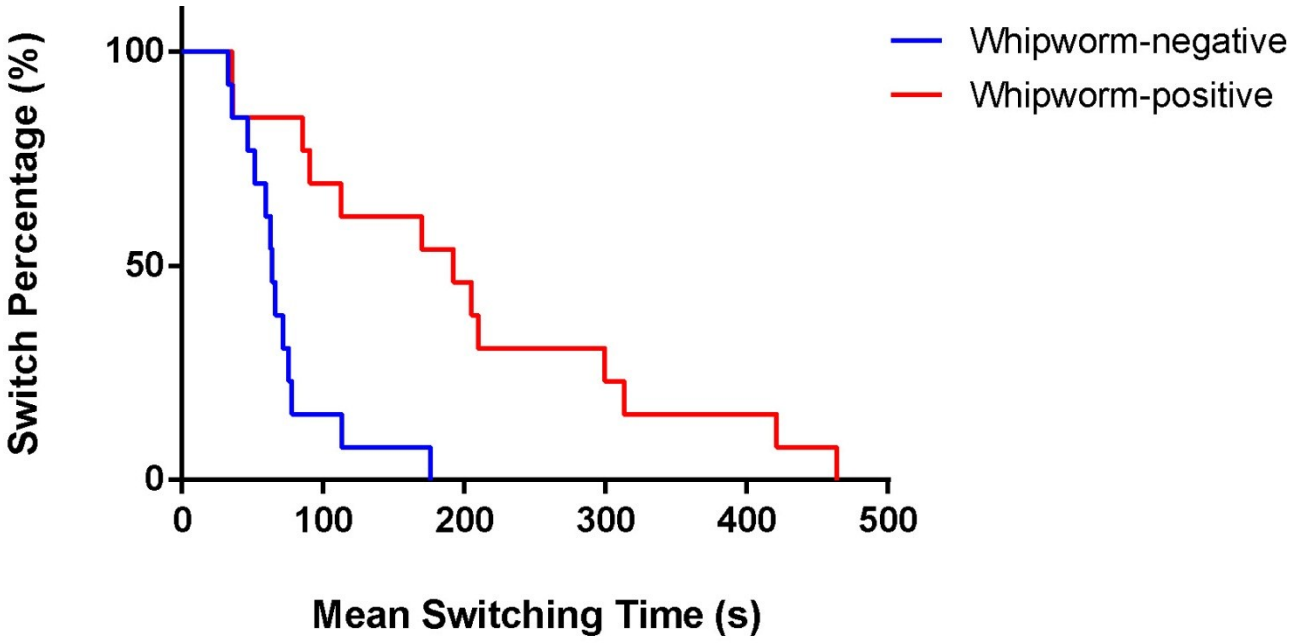

Figure 5.3: **Average duration of behaviour at whipworm-positive and negative intervals.** Kaplan-Meier curves of 14 individuals (eight female, six male) at intervals when negative (blue) and positive (red) for whipworm eggs in feces. Each curve shows the mean time (seconds) required for an individual to switch behaviour in a 30 minute focal period, with the percentage of individuals that have not switched behaviour shown on the Y axis.

CHAPTER 6

General Conclusions

6.1 General Conclusions

Parasitic diseases are increasingly recognized as important to both public health and wildlife conservation because their burden is predicted to greaten with projected global changes. The parasites investigated in this thesis are transmitted through the environment and through vectors, which are both modes of transmission expected to be considerably influenced by a warming climate (Altizer *et al.* 2013). Indeed, recent empirical studies re-affirm early predictions that parasites with environmental stages and vectors are likely to increase in abundance and severity with warming (Harvell *et al.* 2002; Garamszegi 2011; Zamora-Vilchis *et al.* 2012). In ecological communities, climate change can therefore generate systemic imbalances that result in the loss of some host-parasite interactions but the gain or exacerbation of many others (Altizer *et al.* 2013).

Our world is also becoming increasingly connected. Not only are people and products travelling greater distances and more frequently, but we are also moving closer to wildlife habitats. Continued human encroachment on these areas will inevitably elevate exposure to parasites and facilitate the transmission of new diseases between people and wildlife.

In the face of these global changes, a number of studies have called for empirical data from wildlife populations, to be used to interpret macro-ecological patterns and to project into the future (Altizer *et al.* 2003; Thompson 2013). This thesis was an attempt to answer such a call. Below, I highlight the key findings of my thesis and their contributions to the field of host-parasite interactions.

6.1.1 There is Much to Be Discovered

Nearly 30 years ago, a conservative estimate predicted that parasites comprise just under half of all biodiversity on earth (Toft 1986). Today, we are still discovering and categorizing new parasite species. I investigated the taxonomy of four parasites, and from three, I discovered multiple lineages that were distinct from anything genetically characterized previously. Among soil transmitted helminths, I found one *Oesophagostomum* lineage that was transmissible between humans and several species of monkey, and one *Trichuris* lineage that was found only in humans. Both showed enough genetic variation to be new species, if the DNA regions examined are representative of overall biology. Among blood parasites, I characterized four

lineages of *Hepatocystis* that did not match published sequences. Unfortunately, species-level designations have not been assigned to *Hepatocystis* DNA sequences, which makes it difficult to ascertain if these lineages represent distinct species. Nevertheless, these results highlight the incredible diversity of "neglected" parasites that have evaded discovery. This is despite this work being conducted in a well-established research locale, and on wild primates, our closest evolutionary relatives and arguably the best-studied host order. These results suggest that even with tremendous advances in the fields of molecular biology and parasitology, we are still underestimating true parasite biodiversity. However, the domain of host-parasite ecology has moved towards synthesis of already available data. This is likely to be problematic if we are discovering that previous characterizations of parasite diversity are inaccurate or incomplete.

6.1.2 Parasite Transmission and Host Range

At the outset of my PhD, I predicted that soil transmitted helminths, which have relatively slow generation times and complex physiology, are more likely to be specialist parasites because of their life history, which would limit adaptation to new hosts. These predictions were based on comparative results showing that the majority of primate parasites are species-specific (Pedersen *et al.* 2005). However, the results from two genera of soil transmitted helminths suggest the contrary – one or more lineage of each helminth appeared capable of infecting a number of distantly related primates in the community, as well as humans. While opposite of theoretical expectations based on parasite traits (Pedersen *et al.* 2005), my results agree with expectations based on parasite mode of transmission. Both *Oesophagostomum* and *Trichuris* are environmentally transmitted, which means that their infective stages are likely to encounter many potential hosts that move through contaminated environment (Woolhouse *et al.* 2001). Therefore, if adaptation to new hosts is not as challenging as previously thought, host range may be determined largely by the opportunity for infection. In addition, while there are likely large differences between the gastrointestinal systems of the Old World primates examined in this thesis, their physiology may be similar enough that cross-transmission is feasible. In particular, parasites that infect organ systems like helminths may not require as much specificity as, for example, a virus requiring receptor recognition to enter a cell.

Contrasting with soil transmitted helminths, theory predicts that blood parasites, which have relatively fast generation times and encounter many potential hosts through their vector, are more likely to be generalist parasites (Woolhouse *et al.* 2001). However, my findings were again contrary to predictions – most lineages of *Hepatocystis* and all species of *Plasmodium* identified were species-specific. A study examining 29 primate malaria (*Plasmodium*) species also expected to find broad host ranges among blood parasites, but instead found a gradient of specificity, ranging from extreme specialists to extreme generalists (Garamszegi 2009). However, the study found that transmission of malaria between host species was non-random; it was strongly constrained by the phylogenetic relatedness of hosts (Garamszegi 2009).

Previous comparative work has also shown that the phylogenetic relatedness of hosts most strongly influences patterns of parasite transmission (Davies & Pedersen 2008; Cooper *et al.* 2012). However, none of the parasites that I examined yielded unambiguous indication of transmission by phylogeny or spatial overlap (geography). One *Oesophagostomum* lineage appeared to be phylogenetically constrained to the monkey Family (Cercopithecidae), but the other two lineages did not follow similar patterns. Neither *Trichuris* nor *Hepatocystis* showed patterns of transmission matching host phylogeny, and had host ranges that were either extremely generalist (*Trichuris*) or extremely specialist (*Hepatocystis*) within the community. At least with respect to *Trichuris*, examining a greater diversity of potential hosts, including nocturnal primates, carnivores and ungulates, may help clarify transmission patterns.

While these results do not negate the importance of identifying broad patterns of parasite transmission, they highlight the complexity of dynamics at the community level. They also suggest that macro-ecological transmission patterns might not scale; while useful for predicting dynamics at large scales, they may be less useful for predicting them locally, where potentially idiosyncratic events are also influencing dynamics.

6.1.3 Host Range by Lineage

In a 2001 article on the relative advantages of parasite specialism and generalism, the authors concluded, *"the evolutionary advantages and disadvantages of generalism are so finely balanced that even closely related pathogens can have very different host range sizes"*

(Woolhouse et al. 2001). My findings in Chapters 2 and 3 fit this statement precisely. In both *Oesophagostomum* and *Trichuris*, I identified multiple lineages with different host ranges. *Trichuris* provided the most extreme example, with two lineages that were only found in humans or colobines, while a third was found in all primates examined. Moreover, *Oesophagostomum* and *Trichuris* have similar life histories, modes of transmission, and the same pool of potential hosts in Kibale. Yet the patterns of transmission in each were unique and unpredictable based on theory alone. These results suggest that the dynamics involved in a particular parasite's transmission are affected by a number of factors, likely including fine-scale or subtle processes like competition, physical adaptations, and resource availability.

6.1.4 Costs of Parasitism

My thesis research focuses on neglected tropical parasites that (with the exception of *Plasmodium*) typically do not kill their hosts. Some of these parasites have known pathology in captivity. For example, captive gorillas suffer similar symptoms to humans severely infected with *Oesophagostomum*, including abdominal discomfort and intestinal lesions caused by parasite larvae (Sleeman *et al.* 2000; Guillot *et al.* 2011). However, wild gorillas appear to tolerate infection, and it is believed that pathology only manifests when the host is immunocompromised or is infected by abnormally high intensities of parasites induced by captivity (Sleeman *et al.* 2000). Chapter 5 of my thesis explored the possibility that wildlife also suffer the costs of non-lethal parasitism. While whipworm did not result in overt pathology, infection corresponded with changes in behaviour including apparent lethargy, which suggest that red colobus are clinically sensitive to infection. In Chapter 3, I characterized the lineages of whipworm infecting red colobus and other primates. All but one red colobus was infected with a broadly transmissible whipworm lineage. The results of Chapters 3 and 5 together suggest that most whipworm infections in red colobus are caused by a multi-host parasite that has measurable effects. This parasite can infect all diurnal primate species in the community (as well as adjacent humans), which suggests that shifting host dynamics within this system could have direct effects on red colobus health. This is important in changing global conditions. In isolation, non-lethal parasites such as whipworm may only have small negative effects. However, with climate creating favorable conditions for disease and human-mediated changes in land-use causing

susceptible hosts to interact more intensely, multi-host parasites with non-lethal consequences may add to a “perfect storm” of conditions that push species towards extinction.

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APPENDICES

Appendix A: Co-Infection, *Oesophagostomum* (Ch. 2) and *Trichuris* (Ch. 3)

Since the same fecal samples were used to screen both *Oesophagostomum* (Ch. 2) and *Trichuris* (Ch. 3), I could directly compare the prevalence of these two parasites and identify specific samples co-infected with both helminths.

A.1 Prevalence

Overall, the community-wide prevalence of *Oesophagostomum* (70%) was double that of *Trichuris* (34%), although the prevalence of both are considered high (Figure A1). The prevalence of these two parasites varied considerably by species. For example, nearly all baboons were positive for both *Oesophagostomum* and *Trichuris*. However, fewer than half of all red colobus or humans were infected by either parasite (Figure A1; Table A1).

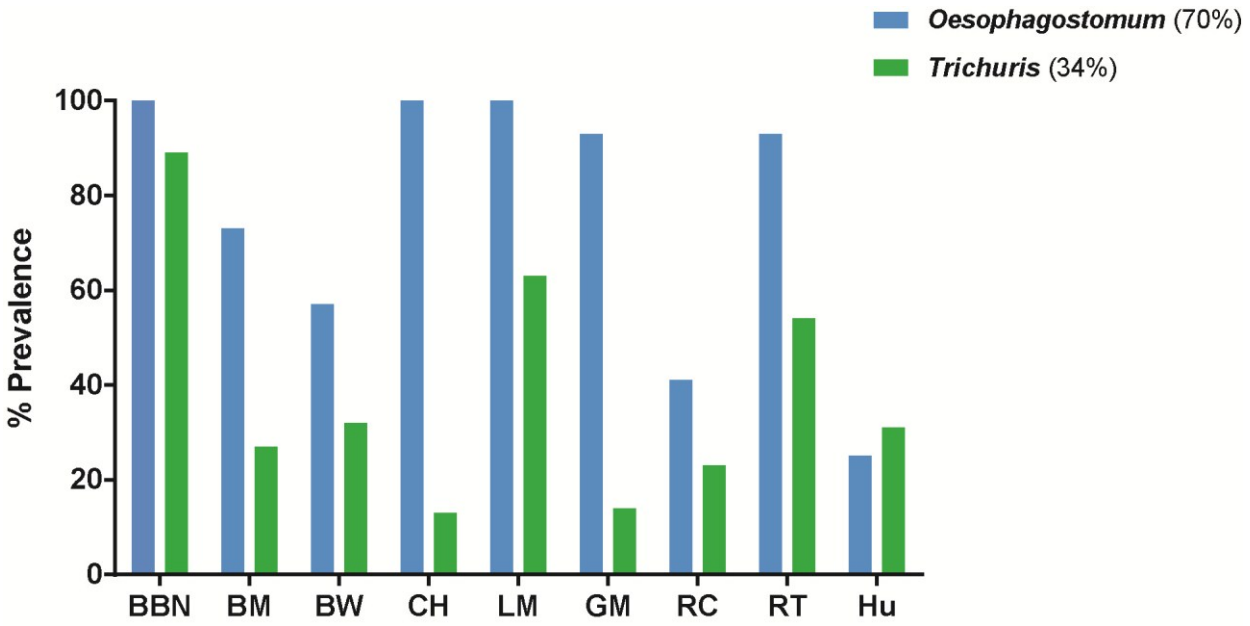

Figure A1: Prevalence of *Oesophagostomum* and *Trichuris*. From 318 samples across all diurnal primate species within the Kanyawara study area of Kibale National Park. Host species abbreviations are: BBN: Olive baboon, BM = Blue monkey, BW = Black-and-white colobus, CH = Chimpanzee, LM = L'hoest monkey, RC = Red colobus, RT = Red-tailed guenon, and Hu = Human.

A.2 Co-infection

By examining individual samples, I could also determine the prevalence of co-infection in the primate community. Overall, nearly half of all species were singly infected with *Oesophagostomum*. However, one-quarter of samples were co-infected with both *Oesophagostomum* and *Trichuris* (Table A1). Combinations of soil transmitted helminth infections varied substantially by species. For example, chimpanzees and grey-cheeked mangabeys were most often singly infected with *Oesophagostomum*. In contrast, baboons and red-tailed guenons were frequently co-infected with *Oesophagostomum* and *Trichuris*. Some species, including chimpanzees, l'hoest monkeys, and baboons did not have any samples that were uninfected. Other species, however, had high proportions of uninfected samples – specifically red colobus and humans (Table A1). In order to determine if being infected with *Oesophagostomum* was associated with a higher probability of *Trichuris* infection or *vice versa*, I conducted an odds ratio calculation, which tests the association of two properties in a population. The results were not significant (odds ratio = 1.72, Fisher's exact $p > 0.05$), suggesting that infection with one of these two parasites neither facilitates nor hinders infection by the other.

In order to visually assess spatial patterns in infection, I used the Global Positioning System (GPS) points recorded at the time of fecal sample collection to map the physical location of each primate (including humans) and their infection status at the time of collection. Unfortunately, no spatial patterns are obviously discernible, and since many of these primate species are known to range throughout the Kanyawara region, no statistical analyses were performed on these data (Figure A2).

Table A1: Co-infections of *Oesophagostomum* and *Trichuris*.

Host	*N*	Uninfected (%)	*Oesoph*. only (%)	*Trichuris* only (%)	*Oesoph.* & *Trichuris* (%)
BM	33	6 (18.2)	18 (54.5)	3 (9.1)	6 (18.2)
BW	37	9 (24.3)	15 (40.5)	7 (18.9)	6 (16.2)
CH	30	0 (0)	26 (86.7)	0 (0)	4 (13.3)
GM	42	3 (7.1)	33 (78.6)	0 (0)	6 (14.3)
HU	36	19 (52.8)	6 (16.7)	8 (22.2)	3 (8.3)
LM	8	0 (0)	3 (37.5)	0 (0)	5 (62.5)
OB	27	0 (0)	3 (11.1)	0 (0)	24 (88.9)
RC	64	30 (46.9)	19 (29.7)	8 (12.5)	7 (10.9)
RT	41	3 (7.3)	16 (39.0)	0 (0)	22 (53.6)
Total	318	70 (22.0)	139 (43.7)	26 (8.2)	83 (26.1)

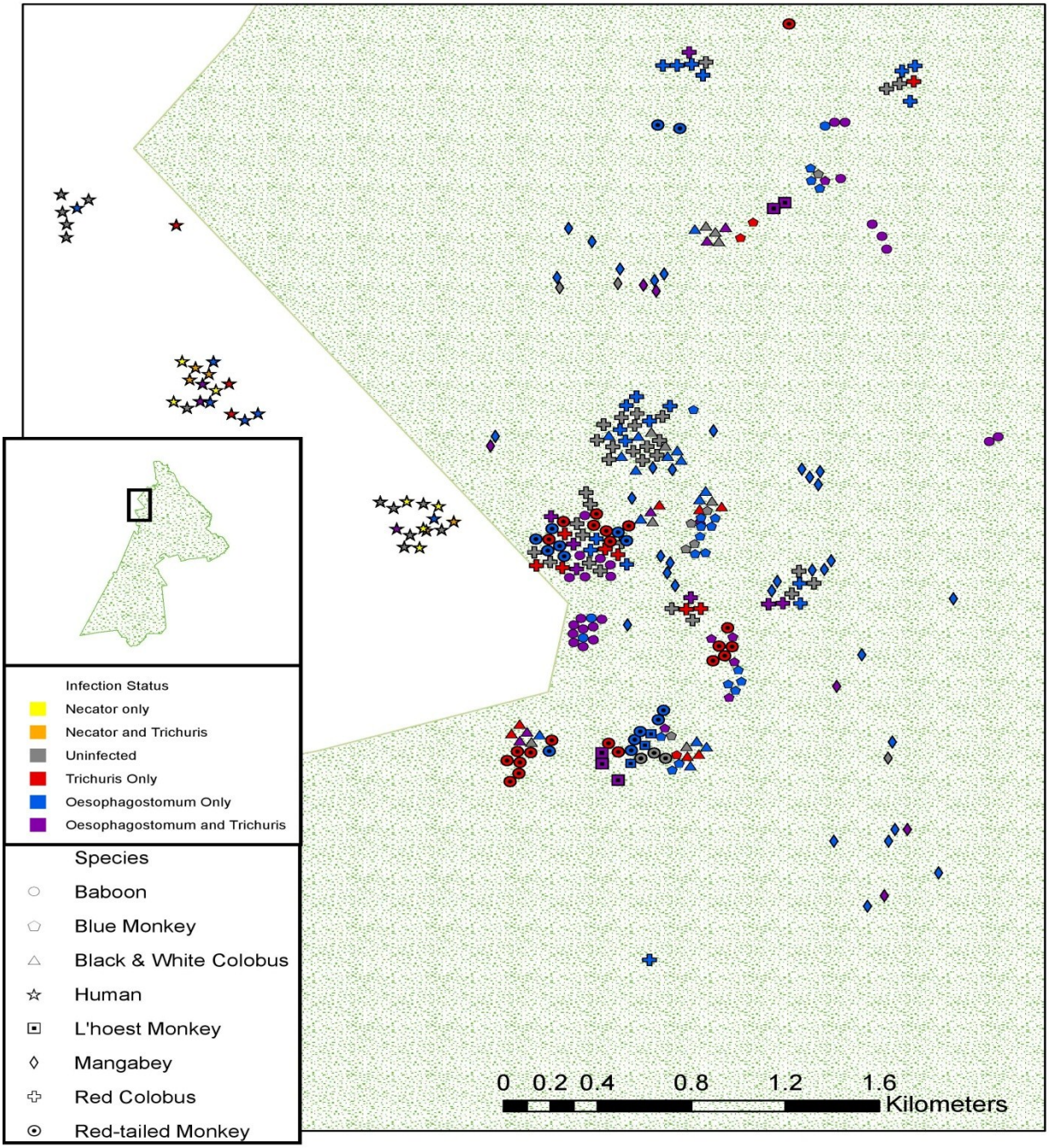

Figure A2: Spatial patterns of primate helminth infection in Kanyawara. Chimpanzees are not included in this map because their samples were collected from a south western region of Kibale National Park. Results include *Necator* (hookworm), another soil transmitted helminth detected only in human samples.

Appendix B: Supplementary Material for Chapter 5

B.1 Supplementary Methods

B.1.1 Determining phylogenetic changes in feeding

Since plant compounds used for medicinal purposes are often evolutionarily conserved, we explored how red colobus foraging choices changed in phylogenetic structure between whipworm-positive and negative intervals. The plant families eaten during this study were used to reconstruct phylogenetic relationships in Phylomatic v.3 using the R20120829 backbone tree for plants, with branch lengths dated in Phylocom v.4.2. (Webb *et al*. 2008). Net relatedness index (NRI) was calculated in the picante library (Kembel *et al*. 2010), where positive NRI values indicates phylogenetic clustering (*i.e.*, red colobus preferentially feed on closely related plant species), and negative NRI values indicates phylogenetic over-dispersion (*i.e.*, red colobus preferentially feed on distantly related plant species). Mean pairwise distance (MPD) was weighted by the proportion of time red colobus spent feeding on the respective plant family, and statistical significance assessed by comparison to 1000 randomly assembled communities (Webb *et al*. 2002).

B.2 Supplementary Tables

Table B1: Sample size of the five behaviours included in analysis. The number of activity data recordings per behaviour, and the number of individuals with data coverage.

Activity	*N*	% Activity	# INDVS
Copulate	100	3.30	25
Feed	1460	48.15	43
Groom	218	7.19	38
Move	215	7.09	40
Rest	1039	34.27	43
TOTAL	3032		43

Table B2: Plant species in the Fabaceae (Leguminosae) family consumed by red colobus at whipworm-positive and negative intervals. Bolded values indicate statistically significant differences in plant species consumption between infection statuses, assessed using binomial tests.

Plant Species	% Consumed	
	Negative	Positive
Acacia bravespica	7.27	4.17
Acacia hockii	3.64	1.67
Acrocarpus sp.	0.91	0.42
Albizia grandbracteata	**19.54**	**37.08**
Albizia gummifera	**4.09**	**7.92**
Erythrina abyssinica	1.36	0.83
Millettia dura	15.00	9.17
Newtonia bucchanani	48.18	38.75
TOTAL *N* Fabaceae	220	240
TOTAL *N* (All Plant Families)	945	760

B.3 Supplementary Figures

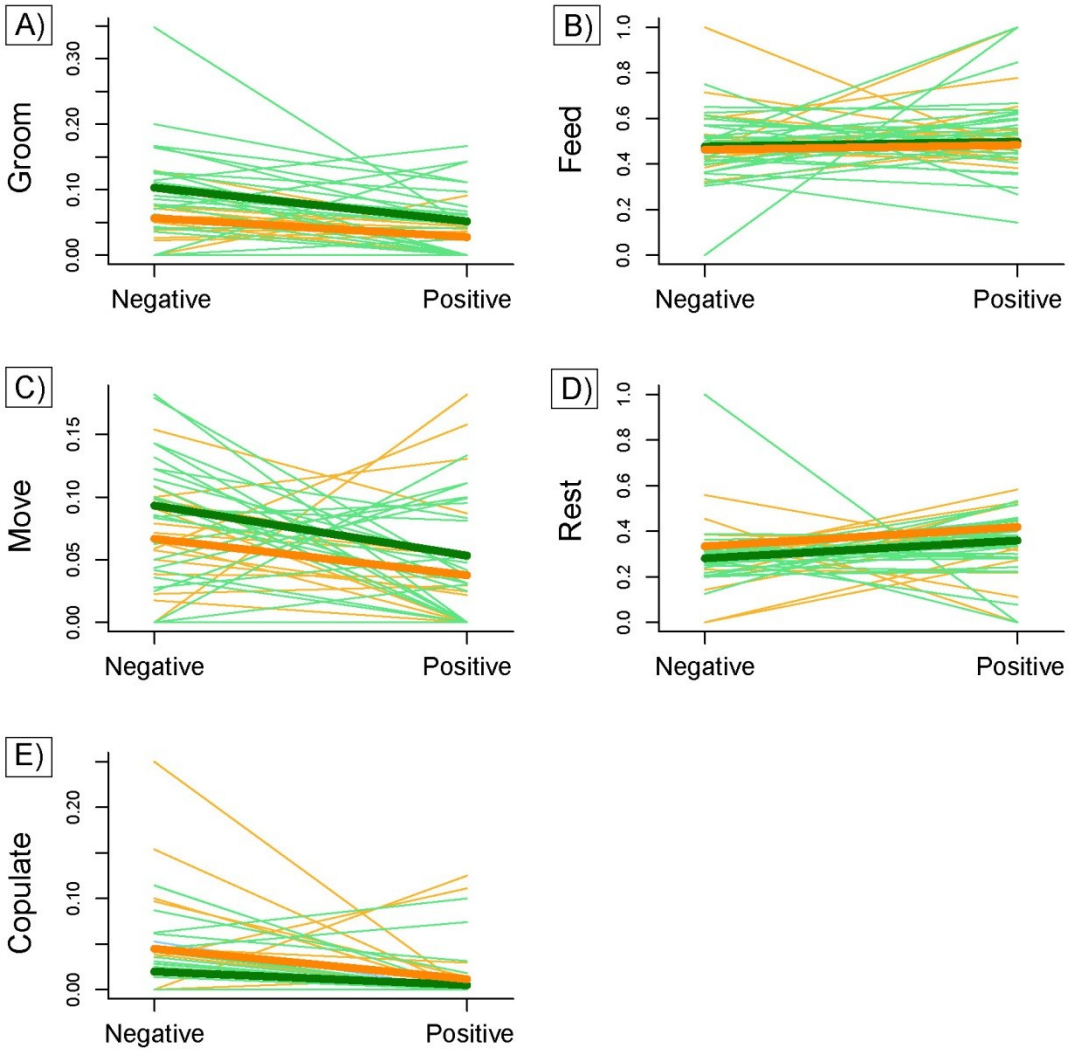

Figure B1: **Relationship between whipworm infection status and activity of red colobus.** Generalized linear mixed models (GLMMs) were used to test how the proportion of observations spent on each of five behaviours (A-E) was affected by (i) sex and (ii) infection status (positive or negative for whipworm eggs in feces). Individual identity was included as a random effect. Individuals (thin lines) and GLMM model fit (thick lines) are color coded by sex (males = orange, females = green). Details of GLMM results are given in Table 1.

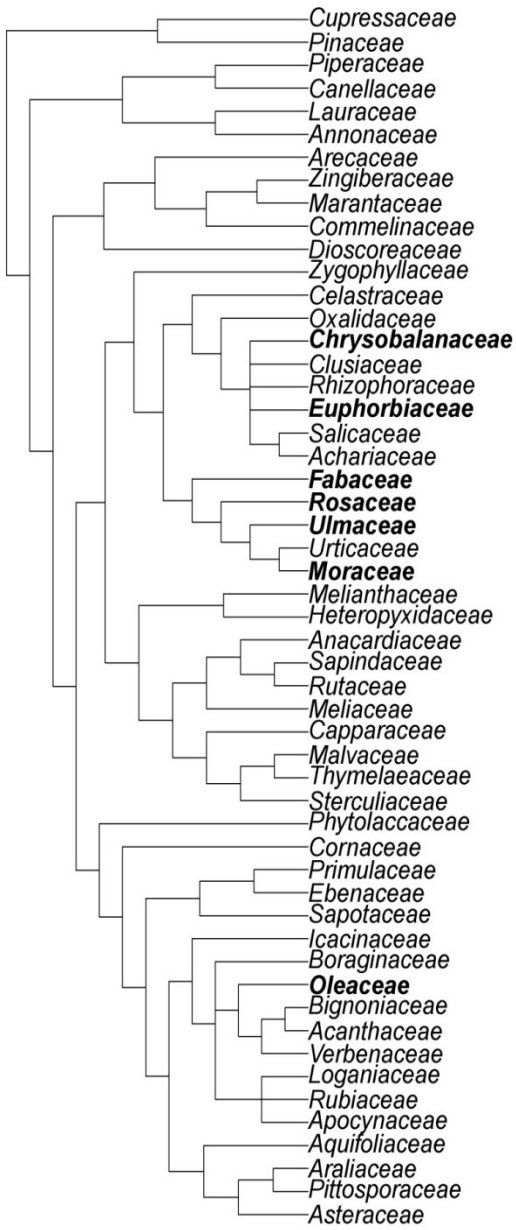

Figure B2: **Phylogenetic relationships of plant families consumed by red colobus monkeys.** Plants consumed by red colobus were classed into plant families whose phylogenetic relationships were reconstructed in Phylomatic using the R20120829 backbone tree for plants. Branch lengths were dated in Phylocom. Plant families consumed in ≥ 5% of all feeding events are bolded irrespective of infection status, since red colobus did not change the phylogenetic structure of their feeding choices between whipworm-positive and negative intervals.

B.4 References

Kembel S.W., Cowan, P.D., Helmus, M.R., Cornwell, W.K., Morlon, H., Ackerly, D.D., Blomberg, S.P., and Webb, C.O. (2010). Picante: R tools for integrating phylogenies and ecology. *Bioinformatics*, 26, 1463-1464.

Webb C.O., Ackerly D.D., McPeek M.A., Donoghue M.J. (2002). Phylogenies and community ecology. *Annual Review of Ecology and Systematics,* 33, 475-505.

Webb C.O., Ackerly D.D., Kembel S.W. (2008). Phylocom: software for the analysis of phylogenetic community structure and trait evolution. *Bioinformatics*, 24, 2098 - 2100.