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_____ _____

Dylan C. Grippi Date

Within-host Ecology: Parasitism over a Changing Landscape

By

Dylan C. Grippi
Master of Science

Graduate Division of Biological and Biomedical Science
Population Biology, Ecology, and Evolution

_____

Jacobus C. de Roode, Ph.D.
Advisor

_____

Nicole M. Gerardo, Ph.D.
Committee Member

_____

Todd A. Schlenke, Ph.D.
Committee Member

_____

Venkatachalam Udhayakumar, Ph.D.
Committee Member

Accepted:

_____

Lisa A. Tedesco, Ph.D.
Dean of the James T. Laney School of Graduate Studies

_____

Date

Within-host Ecology: Parasitism over a Changing Landscape

By

Dylan C. Grippi
B.S., University of Arizona, 2008

Advisor: Jacobus C. de Roode

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Abstract

Within-host Ecology: Parasitism over a Changing Landscape
By Dylan C. Grippi

The abundance of within-host ecological interactions is important for framing questions that verify classical examples from the literature. Investigating how these parasite-parasite interactions can be manipulated through their host requires understanding how changes to the host impacts its parasites, and what external forces cause these changes. Habitat disturbance and fragmentation are two such anthropogenic changes than can impact host health. How these external forces potentially impact within-host ecology are explored. As a case study, preliminary data from a small mammal field-collection taken from Kibale National Park, Uganda are examined for two genera of vector-borne haemoparasites, to determine if interactions are altered by habitat disturbance. The aims of this study are to determine parasite prevalence in relation to habitat disturbance, determine if co-infection occurs between these two parasites and, if so, are they interacting, and to evaluate the mechanisms for interaction using ecologically meaningful terms. Shortcomings, necessary considerations, and future works are discussed that will strengthen the field of within-host ecology.

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Within-host ecology: Parasitism over a changing landscape

Dylan C. Grippi

Preface

Ecological interactions in nature are both diverse and vast. Traditional examples have focused on charismatic fauna or other macroscopic organisms (see Begon et al. 1996). These provide excellent instructional tools in the classroom as macroscopic systems are easy to conceptualize. However, focusing on these interactions alone ignores a wealth of study systems that may provide novel solutions to scientific inquiries. If we set our sights to a smaller scale, to within-hosts, a proverbial menagerie of organisms greets us with their own diverse array of ecologically meaningful interactions. Study of within-host systems is also critical in understanding novel disease emergence and how pathogens evolve both virulence and drug-resistance. As will soon become apparent, a combination of studies that investigate hosts, organisms within hosts, and the large-scale biotic and abiotic forces that govern them, will be necessary in the study of host-parasite ecology and evolution.

Ecology is governed by a combination of species-species interactions and interaction with the natural environment. For within-host systems this is complicated, as environments for parasites are defined by both their host and their host's habitat. The host's habitat is important for within-host systems because ecological interactions that a host encounters and the resources that are available from its habitat alter the host's fitness and health. Changes to this habitat can negatively affect the host's health through stress (Martínez-Mota 2007) and resource limitation (Chapman et al. 2006). While

habitat change can be a natural process, human induced disturbance and fragmentation is on the rise and is often abrupt (Vaz et al. 2007, Chapman et al. 2005,), causing sweeping changes to communities and ecosystems. Such changes have unpredictable outcomes to both species richness and the health of surviving populations (Chapman et al. 2005, Cosson et al. 1999). As might be expected, this can impair a host's ability to fight off infection and is often observed by increased disease prevalence in areas of habitat disturbance for numerous parasite genera with various pathways of infection and pathogenesis (gastro-intestinal, vector-borne) (Cottontail et al. 2009, Vaz et al. 2007, Gillespie et al. 2008, Gillespie and Chapman 2006). As a result, I anticipate that as habitats change, and hosts suffer the consequences, the within-host environment for parasites will change as well. How this change impacts the ecological interactions between parasites is yet unknown and will likely depend upon how resource availability within the host and opportunity for co-infection between different parasites is altered. Since disease prevalence is often positively associated with habitat disturbance, the opportunity for parasites to encounter each other within a single host should also be increased.

When co-infection events like the above described occur, creating a descriptive, resourced based analysis to determine if and how parasites are interacting will be necessary to reveal relationships between external forces on hosts (habitat disturbance) and within-host ecology. Doing so will allow the field to expand and determine not only the effects of habitat disturbance to within-host systems, but also what kinds of disturbance are most important in manipulating these processes.

This thesis aims to establish a functional understanding of the ecological interactions and environmental forces involved in the within-host ecology of natural systems. This will be done through the following goals:

Goal 1: Review relevant ecological interactions from both classical and within-host examples to build resource frameworks for within-host studies.

Goal 2: Review the impact of habitat change through anthropogenic disturbance on hosts and host-parasite interactions to illustrate that host habitat change is important to within-host ecology.

Goal 3: Determine if habitat disturbance influences within-host ecological interaction between parasites.

Goal 4: Discuss and provide recommendations for future research on habitat disturbance effects on within-host ecology.

These goals will be met through the following chapters. Chapter 1 provides reviews on the relevant ecological interactions to within-host systems and on how habitat disturbance effects host-parasite systems. Chapter 2 is the research portion of this thesis and details the study of haemoparasite prevalence of small mammals from field-collected data; with analyses for effect of habitat disturbance on within-host parasite interactions. Chapter 3 concludes this thesis by discussing the complications of studying within-host ecology from field collected data. The final sections will include recommendations for future study design, considerations, and research methods to further extract within-host data from field studies.

Study Organisms

Research for this thesis was conducted on a field-collected sample of small mammal blood-smear slides, which was examined for the prevalence of vector-borne haemoparasites belonging to two genera: *Plasmodium sp*. (agent of Malaria) and *Trypanosoma sp*. (agent of Trypanosomiasis).

Malaria parasites are intracellular/extracellular haemoparasites that represent one of the most important diseases in human history. Human malaria infections are estimated to cause between 300 to 500 million clinically recorded cases annually and up to 2.7 million deaths per year (Ito et al. 2002). Much of what is known about human malaria disease has come from research on *Plasmodium sp*. that infect rodents (Killick-Kendrick and Peters 1978). Research on rodent malarias have produced insight into virulence evolution (Mackinnon and Read 1999) and intraspecific competition within hosts (Bell et al. 2006, Råberg et al. 2006, de Roode et al. 2005).

Trypanosomes are flagellated protozoan parasites that infect diverse clades of organisms and can cause severe zoonotic disease: leishmaniasis, American trypanosomiasis (Chagas' disease), African trypanosomiasis (sleeping sickness), and animal African trypanosomiasis (Nagana). African *Trypanosome sp*. of mammals are extracellular haemoparasites. Trypanosomes have also illustrated intraspecific competition within hosts (Balmer et al. 2009) and have been used in the study of host-parasite interaction over fragmented and disturbed landscapes (Vaz et al. 2007, Cottontail et al. 2009).

These two genera of haemoparasites are ideal for studying within-host interactions in the wild. They both occupy the same host 'environment' (blood and tissues), result in generalized immunosuppression (Askonas and Bancroft 1984, Mendis et al. 1990, Riley E et al. 1988), and cause anemia by either erythrocyte lysis due to reproduction (*Plasmodium sp*.) or auto-antibody and compliment mediated erythrocyte

lysis (*Trypanosoma sp.*) (Rickman and Cox 1979, Amole et al. 1982, Li et al. 2001). Further, classical laboratory studies have shown that plasmodia and trypanosome species show no cross-reactive adaptive immune response (Cox 1972). With this knowledge, several possible mechanisms for within-host interactions are possible for investigation.

Early co-infection studies of these haemoparasites in rats determined that hosts infected by both *P. berghei* and *T. lewisi* had prolonged and increased parasitemia of *P. berghei* when compared to malaria infection alone; trypanosome infections increased after a period of nine days (Hughes and Tatum 1956). This was confirmed in a later study with similar increases in malaria and delayed increased in trypanosome parasitemias (Shmuel et al. 1975). Similar results were found for plasmodium-infected mice given a secondary infection of *T. musculi* (a rodent-specific trypanosome) (Cox 1975). Trypanosomiasis in concomitantly infected mice was found to be enhanced by up to 10 times higher than *T. musculi* infection alone. Since both parasites appear to benefit by the presence of the other, laboratory studies would indicate that concomitant infections result in mutualistic exploitation of the host. Tentatively, I hypothesize that natural infection with *Trypanosoma sp.* will be observed a higher prevalence of *Plasmodium sp.* and vice versa. Caution must be taken, however, as there is a possibility for competitive interaction (Figure 2.1) that may not be apparent in controlled laboratory studies that provide optimal conditions for the host. Sub-optimal natural conditions or disturbed habitats may limit host resources (erythrocytes, iron/hemoglobin, oxygen, etc) and result in the observation of competitive interactions.

Study Site

Samples were collected over a range of habitat disturbance to analyze hypotheses on disturbance effect on within-host interactions. The field location, Kibale National

Park, Uganda and surrounding areas, were used as they have a long and established history of serving as a natural laboratory for studying the ecological impact of habitat disturbance and forest fragmentation (Struhsaker 1997, Onderdonk and Chapman 2000, Chapman et al. 2005, Gillespie and Chapman 2006, Chapman et al. 2006). Further more, field study of rodent malaria parasites necessarily needs to be done on the continent of Africa, as their current known distribution remains isolated from the rest of the world (Killick-Kendrick and Peters 1978). As previous studies have concluded that prevalence of *Trypanosoma sp*. infections are increased in areas of habitat fragmentation (Vaz et al. 2007, Cottontail et al. 2009), I hypothesize that habitat disturbance will be an important factor in *Trypanosoma sp*. prevalence. Following my previous hypothesis on co-infection, this also indicates my expectation that *Plasmodium sp*. will have higher relative prevalence in disturbed habitats.

Chapter 1: Interactions and Disturbance; A Review

Abstract

Ecological interactions are omnipresent throughout all radiations of life and can be manipulated by external forces. Basic pair-wise definitions of interaction have their founding in resources and mechanisms that bring organisms together. Comparisons between traditional and within-host examples are made, with a special emphasis on competition, predation and parasitism, and mutualism. A connection between the fields of within-host ecology and habitat disturbance is made by considering how hosts facilitate environmental interaction with their parasites.

Introduction

Understanding the interactions of parasites within hosts is important to framing ecological questions for these systems. It is critical to establish what these interactions are, what defines them and makes them distinct, since most studies focus on host population level prevalence that may be misinterpreted in the absence of a biological mechanism for interaction. Hosts do not provide perfectly constant environments for parasites to develop in, so it is also important to understand these interactions when considering how changes to the host may alter parasite ecology. Since hosts are a product of their environment, external changes that alter their health will have downstream impacts to parasites carried.

Pair-wise interactions

Biological systems are comprised of numerous populations sharing a landscape of habitats with limited resources. These populations interact when resource acquisition

and location overlap. The result is a diverse array of positive and negative pair-wise interactions. Evidence for these comes from understanding what resources bring populations together, the requirements of populations for these resources, and the mechanism by which they interact. Broadly, pair-wise interactions can be classified into 3 main groupings: competition, predation and parasitism, and mutualism.

Within-host systems, while less traditionally studied for ecological interactions, carry examples of each. When compared to traditional examples, within-host systems illustrate how they can provide a valuable resource in the evaluation of ecological thought. By understanding the resources and pathological mechanisms parasites require and undergo in a host, we can build expectations on whether competition between different parasites is likely.

Competition

Competition forms the foundation of most interactions between organisms as none live in complete isolation. Here, competition is considered as an interaction between two populations that require the use of a shared resource. This resource is limited in availability and interaction between competitors to gather or gain access to it results in a negative effect on each other's density. Resources range from biological requirements (foodstuffs) to physical space (nesting sites). Resources can also concern less tangible concepts such as 'enemy free space' (Holt 1977, 1984, Jeffries & Lawton 1984, 1985); which describes a population's ability to maintain a higher or critical density due to an absence or lower level of predation.

Competitive interactions can occur within or between species. Intraspecific competition, the competition within a particular species of organisms, arises from the similar needs that come between individuals of the same population. Within a particular species, differing genotypes confer variable phenotypic advantages that impose or deny

access to required resources. Interspecific competition on the other hand, occurs between species. This can incur altered population dynamics between competitor species that result in various outcomes of success or failure.

Within-host systems are no exception to this rule and carry evidence for both kinds of competition in both field collected data (Arnot 1998, Mercereau-Puijalon 1996, Daubersies et al. 1996) and laboratory manipulation (de Roode et al. 2005, Råberg et al. 2006, Barrow & Page 2000, L. Sernicola et al. 1999, Lipsitch 1999). Resources for within-host systems, much like those in the classical literature, represent biological requirements and space. There are three general mechanisms of competition that occur: exploitation, interference, and apparent.

Exploitation competition

Competition for resources does not require a direct interaction between competitors; it can be a passive process. Resources used by one population are considered exploited when they have been depressed from the environment and are not available to other populations that may require them. The effected populations suffer from the reduced availability of the resource through a reduction in fitness or survivorship. The evidence for these types interactions comes from close monitoring of an identified resource in relation to competitor population density.

A commonly used example of exploitation competition comes from the laboratory study of freshwater diatoms *Asterionella Formosa* and *Synedra ulna* by Tilman (et al. 1981). The common resource required by both of these species is silicate, used to construct their cell walls. To observe that exploitation competition occurred between these two, population density of both species as well as the impact on silicate usage by each was recorded. When silicate was constantly added to the system, each diatom on its own was able to reach a steady population density. However, *Synedra* was able to reduce

the amount of silicate in solution to a lower level than *Asterionella* in isolation. When these two were allowed to grow together, the effect of this lower silicate level became apparent, as it was lower than *Asterionella* was able to survive on, resulting in its exclusion and *Synedra's* persistence. By preventing *Asterionella* the means to grow and survive, *Synedra's* higher relative resource usage permitted it to succeed in this competition by excluding the other resource user.

This process of resource usage and subsequent competitor exclusion is anticipated to occur for within-host systems, as resources that are critical for parasite survival are often partitioned to specific tissues or cells within the host. An example that is representative of within-host exploitation concerns parasitic intestinal worms of Three-Spined Sticklebacks (Chappell 1969). Natural infections of both the cestode *Proteocephalus filicollis* and acanthocephalan *Neoechinoryhynchus rutili* in the Three-Spined Stickleback result in different parasite distributions within the gut than when occurring alone. Here the parasite species are surmised to be competing over attachment sites within the gut epithelium. Single species infections of both parasites were characterized by broader distributions. Concurrent infections were observed to have different distributions with the *P. fillicollis* population significantly attaching to the anterior intestine while *N. rutili* individuals were attached in the rectum, fewer parasites of both types were observed in the middle ground, the posterior intestine. A 2x2 contingency test was run on *P. fillicollis* and determined that gut distribution was significantly dependent on the presence of the other parasite. This example of competitive exclusion for space does not exclude the possibility that other mechanisms such as interference or apparent competition are involved, but the physical displacement of both parasite populations makes exploitation of resources a likely mechanism.

Making the distinction between exploitation and other mechanisms for competition is difficult, and much room is available to improve empirical studies. Micro-

parasites provide particular difficulty, as the resources they consume or occupy tend to be difficult to quantify in ecologically meaningful ways. Research on the lepidopteran Tea Tortix, *Adoxophyes honmai* (Adho), and competition between two of its viruses, Adho nucleopolyherovirus (NPV) and Adho entomopoxvirus (EPV) provide an example of this (Ishii et al. 2002).

The viruses have a shared requirement of host immune cells/tissue (hemocytes and fat bodies) for replication. Adho NPV rises in density far quicker than Adho EPV when grown in isolation. When grown together NPV will always outcompete EPV if inoculated at the same time, as the latter virus simply cannot develop. When temporally separated however, EPV can develop a similar infection density to that of growth in isolation when it is inoculated first. NPV, when inoculated second, will have greatly reduced density.

While these viruses are both utilizing the same host resource, hemocytes and fat bodies, this does not definitively prove that exploitation is the only or primary mechanism for competition. Direct interference between viruses replication machinery or apparent interactions that are mediated through the hosts immune response are both plausible alternatives. Excluding these alternative explanations will require proper investigation into their pathological cycles and immune reactivity within the host. Direct measurement of resources expected to be under exploitation is also key to within-host studies. Examples of this may include up and coming research concerning resource investment (Wargo et al. 2007) and malaria rosette induction (Mideo 2009).

Interference competition

Competition can, however, take the form of direct interaction between competing parasites. The mechanisms for interference competition can include mechanical and/or chemical means of attack or exclusion conducted by one population or both in an

attempt to garner the most resources for itself. Observation of organism behavior or biology that would implicate direct interaction between organisms is necessary to establish this mechanism of competition.

The classic example of interference from the literature is Connell's barnacle study (Connell 1961, adapted from Begon et al. 1996). This research on physical displacement concerns the Scottish barnacle species *Chthamalus stellatus* and *Balanus balanoides*. Although frequently found together on the same shoreline, adult *Chthamalus* are found in higher intertidal zones while adult *Balanus* are found in the lower zones. Young *Chthamalus* do, however, settle on some of these lower regions, but are unable to last until adulthood.

Connell protected some of the young *Chthamalus* that settled in the *Balanus* region from contact with *Balanus individuals*. These individuals survived just as well as their compatriots in the higher intertidal zone. Direct observation of *Balanus* physically removing, crushing, or smothering *Chthamalus* reaffirmed that *Balanus* was indeed having direct physical competition for development space and was able to outcompete *Chthamalus* in the lower regions of the intertidal zone.

Common mechanisms for within-host interference come from chemical production of antibiotics or other chemical warfare mechanisms. Production of chemicals that manipulate the growth and survivorship of others, called allelopathy, has also been observed in plants (Begon et al. 2006) but its capacity is maximized by within-host bacteria. A strong example of this comes from bacteria that colonize the upper respiratory tract of hosts (Selva et al. 2009). Both *Streptococcus pneumoniae* and *Staphylococcus aureus* inhabit these regions. *S. pnuemoniae* produces hydrogen peroxide compounds that have no effect on it, but are well known to induce lysogenic responses in other bacteria by the SOS pathway (Goerlich et al. 1989). *S. aureus* is no exception and all lysogenic strains, which carry the same intact SOS pathway, undergo

lethal lysis when exposed to hydrogen peroxide producing *S. pnuemoniae*. By doing so, *S. pnuemoniae* is able to proliferate to higher densities as more resources will be at that population's disposal. Direct measurement of these hydrogen peroxide compounds provides evidence that this competition is facilitated by interference.

Such examples of interference competition within-hosts are potentially the easiest to establish, as they require a direct interaction between organisms. Observation of interference competition is abundant and includes a wealth of examples: bacteriocin production by bacteria to kill closely related bacteria that are competing for resources (Riley & Wertz 2002) and anti-microbial production by fungi (Arnold et al. 2003, Mejia et al. 2008). Bacteriocin usage may be one of the most common mechanisms for within-host competition between bacteria, as some produce multiple kinds that can have a variable range of killing (Riley M et al. 2003). Indeed, these natural products have been observed to be responsible for both intraspecific (Kirkup & Riley 2004) and interspecific interference (Massey et al. 2004).

Apparent competition

The final type of competition considered is much less tangible than direct exploitation of resources or interference between competitors. Apparent competition occurs in cases where one organism benefits through the loss of a competitor via indirect means, often mediated through a predator or other organism. This is traditionally thought to occur via competition for 'enemy-free space' (Holt 1977, 1984, Jeffries & Lawton 1984, 1985) or other indirect benefits.

One of the few traditional examples of apparent competition involved 2 groups of prey and 3 predators of a shoreline ecosystem (Schmitt 1987). The first group of prey included 3 species of mobile gastropods while the other included 2 species of sessile bivalves. Predators of these two groups, which showed preference to preying on the

bivalves, included a lobster, an octopus, and a whelk. Presence of either prey group was inversely correlated to the other, but there was no evidence of a shared food source (exploitation) or direct exclusion (interference).

Interestingly, areas with high levels relief from predation, those with increased numbers of hiding spots, were dominated by bivalves and predators and only had nominal numbers of gastropods. Areas of low relief had no bivalves but high numbers of gastropods. When bivalves were experimentally placed in the low relief areas, predator numbers increased in these areas, resulting in higher predation on and reduced density of gastropods. High relief sites with higher than normal gastropod numbers were also characterized by increased predation and death rates of experimentally introduced bivalves, as compared to normal density high relief locations. Thus, the increased number of either prey species in an environment negatively impacts the other prey species, producing a competition through the predator for space with reduced predator numbers.

As this example of apparent competition illustrates, pair-wise interactions that work through proxy organisms can produce ecologically meaningful understanding to otherwise complex systems. It is here that within-host ecology may gain the most new insights as parasites can be considered to interact through host biological responses, such as the immune system. This will be important in intraspecific competition studies, as parasite strains will likely have similar biology that is recognizable to the immune system. It should be anticipated that biological responses hosts make to parasites potentially can have carry over to other parasites, and through the length of evolutionary history, these responses have the potential to be co-opted by parasites as a means to get ahead of the competition.

Rodent Malaria, *Plasmodium chabaudi*, studies have provided one of the strongest and earliest examples of immune system mediated apparent competition

(Raberg et al. 2006). This was accomplished by studying virulent and avirulent clones infecting both immunodeficient and immunocompetent mice hosts. The avirulent clone experienced a lower parasite density in immunocompetent mice as compared to the virulent type in mixed infections. Very little of the reduction of avirulent clones was removed in immunodeficient mice in mixed infections. All other things being equal, as these were genetically equivalent mice (competence was induced by the reintroduction of T cells), the avirulent clone suffered from immune mediated apparent competition when in the presence of more virulent counterparts. Although evidence for immune mediation is very weak and other mechanisms that separate these clones' ability to compete, i.e. how their virulence effects resource consumption are possible, it provides a point from which to further examine the role host response has to within-host interactions.

Predation and parasitism

Counterparts to competitive interactions, predation and parasitism occur when one organism utilizes another as a resource and benefits from its consumption (either in whole or in part). Evidence for these interactions will be rather explicit, when the predator or parasite gains from the loss of an associated prey or host. Although considered much more rare than competitive interactions within a host, forms of predation and parasitism do occur between within-host organisms. Strikingly, viruses can be parasitized by other viruses. In such cases as Adenovirus and Adeno-associated virus, the virus's replication machinery are co-opted within a host cell (Casto et al. 1967, Parks et al. 1968), while other examples exist where the mimivirus, a large virus that only has been found within certain amoeba, has its own virus, Sputnik, that cannot replicate outside of the mimivirus replication structure (La Scola et al. 2008).

Mutualism

Not all interactions between organisms necessarily require a negative effect to be placed on a participant. Mutualisms occur when both members in the interaction gain some beneficial advantage that outweighs the cost. Classic examples from nature include lichens that grow on trees and the fantastic variety of pollinator species and their associated plants. Within-host systems likewise provide a plethora of mutualisms between parasites and symbionts.

Mutualistic interactions are considered for within-host systems on two different levels, between symbiont and host as well as between different parasites living in a host. It is important to establish this distinction as the different interactions result in different ecological mechanisms and selection pressures. One can imagine a symbiont of the host coming into competition with parasites that would otherwise reduce host health and indirectly (or directly) lower fitness of the symbiont. Different ecological interactions would result from two different parasites mutualisticly exploiting a host.

Such an example occurs between species of *Bacillus* bacteria, *B. thuringiensis* and *B. cereus*, which colonize the gut of the Diamondback Moth (Raymond et al. 2008, Broderick et al. 2000). Antibiotic producing strains of either *B. thuringiensis* or *B. cereus* are shown to result in synergistic growth of non-antibiotic producing strains in the moth's gut as compared to growth in the absence of the antibiotic producing bacteria. This occurs both within each species and between them. These results are supported by findings and ideas that these antibiotics reduce the abundance of host commensal gut bacteria, and enabling invasion by the *Bacillus* genus.

Co-infection: Competition and the Host

Unlike experimental manipulations in the laboratory, natural organisms rarely play host to just one parasite (Read & Taylor 2001). The diversity of infection for a host spans both within single species (*intraspecific variation*) and across taxa (*interspecific variation*). It is easy to imagine then that each of these numerous and diverse inhabitants of a within-host system have their own requirements and establish interactions within the host. Single hosts thus, are likely to have parasites that exhibit the full gamut of pair-wise interactions discussed previously. What then happens when multiple interactions occur at once? Variable and sometimes unexpected outcomes are a product of this natural complexity.

Taking a step back from within-host systems, we have seen this level of complexity previously, or at least the silhouette of it, in the example of apparent competition between bivalves and gastropods (Schmitt 1987). Here the prey groups were separated; there was no measurable competition for physical resources, but both felt density depression through the predator species. Thus, the individual interactions between the prey and predator species were insufficient to explain the resultant dynamics. Apparent competition inherently carries this property, as competition is mediated through a third party. Apparent competition is not alone in establishing complex interactions, as resource competition is important as well.

A charismatic example of this involves wildlife of the African savannah: Plains Zebra, Wildebeast, and their predators (Lions and Hyena) (Grange & Duncan 2006). Ungulates represent a large portion of African wildlife and are both diverse and abundant. Here, Zebra, which are often found cohabiting with other ungulates of similar size, i.e. Wildebeast, and are rarely the dominant species despite having an effective physiology for nutritional acquisition and drought resistance. This deviation is explained through the co-occurring interaction of predation from Lions and other large predators.

This was empirically shown by prey species abundance in relation to the relative amount of food resources and a metric of lion predation, biomass. Wildebeast abundance was found to be closely associated with resources while Zebra limitations were associated with predation. Areas with lower abundance of lions were characterized with greater numbers of Zebra.

Bringing focus back to within-host systems we see similar levels of complexity with only slightly changed participants. Graham (2008) conducted a meta-analysis of helminth and microparasite (malaria) infections to determine how helminths may interact with microparasites by manipulating resource availability or immune system response. Although the immune system would be expected to deal with multiple infections that are separated by location or type (Ismail & Bretscher 1999), induction of general responses, such as signaling cytokines, have much broader effects over the entire host system (Germain 2001, Mohrs et al. 2005). Indeed, helminths are shown to induce cytokine responses that are also important in microparasite infections (Cox 2001, Abbas et al. 1996). Graham was able to illustrate that the result of each system depended on the resource requirement for red blood cells (RBCs) or immune regulation that was manipulated by helminth coinfection. Microparasites performed most poorly when helminth infections induced anemia that limited RBC availability, but gained benefit when helminth infections suppressed inflammatory cytokine responses (cytokine interferon (IFN)-y).

While this manipulation by helminths is made clear, it is uncertain that there is a strong interaction (competition) between helminths and parasites. We can imagine that helminth-induced anemia may be a mechanism of interference competition against microparasites as it reduces the total number of RBCs for microparasites to infect. This allows helminths to maintain host resources at relatively higher levels than expected with co-infection of microparasites and benefit from long-term exploitation that could

otherwise be cut short by severe microparasite infection. The perceived benefits gained by microparasites in hosts with reduced (IFN)-y responses by helminth regulation are even more puzzling. Is this a mutualism, and if so, what benefits do helminths gain from a host with higher microparasite burden? These and other questions remain unanswered by simply considering host interactions in isolation.

Interaction between the host and its parasites may be made most clear when it is reparameterized into ecologically meaningful subdivisions: producer and predator. Doing so provides us with 3 general groups of 'organisms': producers (host resources), consumers (parasites and symbionts), and predators (host immune system).

In ecological terms, host cells and tissues can be considered producers. Resource acquisition in all natural systems is limited; those within a host are no different. Limited attachment sites, cells to infect (cellular machinery), and metabolic resources are available across host cell and tissue types. These resources are sampled from and competed over by parasites.

The host's immunological response to parasite infection can be partitioned as well and considered as a 'predator'. Host immune systems are complex interacting networks that respond to parasite invasion by specific and general mechanisms. These are further divided into innate (always present) and adaptive immune responses. These responses operate under different time frames: the innate response being rapid and immediate and the adaptive response being slower and more thorough (Janeway et al. 2005) While not all organisms have traditionally defined "adaptive immune systems" (invertebrates, plants, etc) they do respond and can develop acquired immunity (Chisholm et al. 2006, Rolff & Siva-Jothy 2003).

An interesting dichotomy in immune responses comes from inverse up and down regulation of immune capacity by the host to maximize its ability to stave off infection. While this is a strong mechanism for fighting off single infections, multiple parasite

infections can take advantage of the 'holes' left in the host's defense. One of the best examples of this comes from Th1-Th2 tradeoff (activated through CD4+ cells) in mammals (Pederson & Fenton 2006). Here the Th1 response can be stimulated by intracellular viral infections to produce one signaling profile that up-regulates differentiation of cells needed to fight off viral infections (CD8, macrophages, B cells, Natural Killer cells). Th2 response, on the other hand, is primarily stimulated by extracellular antigens, and is geared toward fighting off larger or extracellular parasites. Since both responses operate through the CD4+ cells, neither can run at optimal capacity alongside the other. These 'holes' are apparent in helminth coinfection studies with intracellular *Mycobacterium tuberculosis* infections (Stewart et al. 1999).

How particular parasites in either single or complex infections navigate the host's immune 'predation' is situation and environment specific, as each parasite will have its own mechanism of avoiding destruction. The inability of immune responses to all run at full capacity concurrently permits flexibility to within-host interactions, where depending upon how the host's systems respond, different parasites might grow to higher densities at different points during an infection. The host also suffers from the drawback of not always having a perfect immune response, as it is susceptible to influence by external forces.

The host's health can be influenced by a lack of resources or exposure to stressors. Lack of physiological resources can affect host health through lack of nutrition that can reduce a host's ability to conduct normal functions and impair immunological responses. Environmental stressors can take many forms (crowding, exposure, storms, predation) and result in upregulation of glucocorticoid production in the adrenal gland of mammals (Martinez-Mota 2007, Saplosky et al. 2000). While these are normal responses, if persistent over an extended period of time, they can negatively impact host health through muscle wasting, growth loss, and suppression of the immune system

(Sapolsky and Pulsinelli 1985, McEwen, 2000). Such physiological degradation and immunosuppression can permit greater numbers and variety of parasites to take up residence in a stressed host. This may allow for novel competition or mutualisms to arise within the host as host immunological defenses are impaired and resources may become more readily limited. This kind persistent stress is observed in populations effected by forest fragmentation (Martinez-Mota 2007).

Habitat disturbance and fragmentation

Studies into within-host ecology often concern the interactions within a single host, or a homogenous population of hosts with likewise homogenous populations of parasites. While this assumption will not always implicitly apply to natural systems, where deeper community interactions necessarily need more work to better describe the interplay between within-host and population level components, it does provide a working basis to understand what bottom-up forces influence host populations. Counter to these internal forces, manipulation of host populations by habitat change may radically alter established ecological dynamics for both host and parasite. The study of habitat disturbance and fragmentation illustrates how large-scale environmental alterations can influence natural systems dramatically.

Habitat disturbance and fragmentation have been shown to impact community composition and alter ecological and evolutionary patterns (Sih et al. 2000, Tabarelli et al. 2004, Brooks et al. 1999, Allan et al. 2003, McCallum and Dobson 2002). These anthropogenic perturbations have been shown to have a negative impact on the abundance of forest primary producers (Laurance et al. 1998, 2000) and consumers (Kruess and Tscharntke 1994, Turner 1996, Fahrig 1997, Andren 1994), reducing the overall species diversity (Ferraz et al 2003, Laurance and Bierregaard 1997). Forest

fragmentation is widely considered as one of the largest threats to global biodiversity (Fahrig 2003, Castro and Fernadez 2004).

Laurance and colleagues (2000) were able to definitively illustrate this by studying large canopy trees from the Amazonian rainforest. Before fragmentation of their study site, they found no significant difference between continuous and to-be fragmented forest plots. Following 20 years of data collection, it was found that large canopy tree mortality rates were 40% higher near the edge of established fragments. Such intense perturbation can have long term effects on the sustainability of habitats as these trees are critical resources for associated animal species and serve key ecosystem service roles.

Biodiversity reduction of bird species has also resulted from forest fragmentation (Brooks et al. 1999). Kakamega Forest, the only remaining fragment of Guineo-Congolian rainforest left in Kenya, has undergone increased reduction and fragmentation for the past half century. Bird species losses from historical data sets have allowed researchers to estimate that 50% of total likely extinctions will occur in the first 50 years post fragmentation and isolation. In evolutionary time, this is a huge number of species lost over a very short period and indicates that conservation efforts need to be enacted shortly after habitat disturbance to preserve biodiversity.

Despite knowing that fragmentation and disturbance result in change, the processes by which habitats and species are affected by disturbances are not fully resolved (Gillespie and Chapman 2005, Onderdonk and Chapman 2000). Host-parasite interactions have received increasing attention over the past decade as habitat fragmentation has been recognized to impact animal fitness and health (Martinez-Mota et al. 2007) but still require further investigation. Increased disease prevalence and multi-host parasitism have repeatedly been linked to fragmentation and habitat disturbance (Allan et al. 2003, Gillespie and Chapman 2005, 2008, Vaz et al. 2007,

Cottontail et al. 2009). The impact of increased disease burden may reduce fitness and prevent host organisms from normally functioning in their environment, further intensifying the effect of habitat disturbance and threatening the sustainability of already strained species (Chapman et al. 2005).

Fragmentation and habitat disturbance have increasingly brought wildlife and human populations together, increasing the chances of producing novel zoonoses (Chapman et al. 2005). Diseases between humans and non-human primates illustrate some of the best examples of this. Cross infectivity of malaria parasites between humans and non-human primates illustrates how opportunities arise when host and vector populations overlap, allowing for new strains to develop from previously isolated ones. Recently a human population in Malaysian Borneo tested positive for *Plasmodium knowlesi*, a malaria parasite whose natural hosts are long-tailed and pig-tailed macaques (Singh et al. 2004).

Vector-borne parasite systems seem to experience higher prevalence and longer occurrence as a result of land-use change (deforestation and swamp remediation) (Afrane et al. 2008, Vaz et al. 2007, Cottontail et al. 2009). Across East Africa, recent malaria epidemics appear to have been exacerbated by such disturbances (Lindbalde et al. 2000, Bodker et al. 2003); although these epidemics may also be explained by climate change (Loevinsohn 1994, Martens et al. 1999) and drug resistance (Bodker et al. 2003). Mosquito vector populations responsible for malaria disease transmission have been shown to have a 77.7% increase in vector capacity in relation to deforested locations (Afrane et al. 2008). This is explained by environmental changes to vector habitats that influence biting, reproductive, and survival rates of mosquitoes (Afrane et al. 2005, 2008, Vittor et al. 2006, Rua et al. 2005).

As habitat disturbance and fragmentation can impair host health and functionality, and appear to exhibit higher prevalence of parasite infections, there is a likely downstream effect on within-host systems, changing within-host interactions between parasites.

Within-host ecology and disturbance

While current research has established that habitat disturbance and fragmentation influences the prevalence and impact of parasites on host populations, few have actively explored how these environmental changes impact within-host ecological interactions between parasite strains or species. Previous research has aimed at understanding the effect co-occurrence of parasites over disturbed habits has on host health and population prevalence (Vaz et al. 2007, Cottontail et al. 2009, Gillespie and Chapman 2007) without explicitly exploring implications to within-host ecology. Jolles and colleagues (2008) have come closest to investigating these interactions in a natural setting. In their study of macro- and micro-parasites of free-ranging African Buffalo, they determined that there were strong interactions between gastrointestinal worms and bovine tuberculosis. This interaction was mediated through the host's immune system via a proposed tradeoff of the host's immune resources, resulting in apparent competition between worms and tuberculosis. Similar research needs to be conducted on communities that operate over a range of habitat disturbance.

The starting point for these types of studies necessarily needs to begin with observed prevalence levels in a community of organisms. While messy field collected data may be difficult to link back to within-host interactions, the reward for making connections between naturally observed processes to what can be produced in the laboratory will be bountiful in validating hypotheses for within-host ecology. Evaluation for competition, predation, and mutualism within-hosts in disturbed environments will

require intense observation for interaction followed by hypothesis driven tests that determine the precise resources and mechanism the interaction develops over.

Chapter 2: Vector-borne haemoparasites of small mammals: prevalence in Kibale National Park, Uganda in relation to habitat disturbance.

Abstract

Habitat disturbance has an important role in global biodiversity change and the emergence of infectious disease. This arises from increased interactivity between hosts, vectors, and parasites. Within-host ecology of co-infecting parasites over a range of habitat disturbance is a new line of questioning that needs to be explored. A preliminary collection of small mammal blood smears from Kibale National Park, Uganda (n=103) was obtained for this study to be analyzed for two genera of haemoparasites: *Plasmodium* and *Trypanosoma*. Both are well-established clades used in within-host ecology and evolution research. 59.2% (N=61) were infected with haemoparasites. 48.5%(N=50) were infected with *Plasmodium sp*. while 17.5%(N=18) were infected with *Trypanosoma sp*. 11.5%(N=7) of infected hosts had concomitant infections by both genera. Novel occurrence of *Plasmodium sp*. was observed in several family Muridae genera. No statistically significant values were obtained for comparison of parasite prevalence over habitat disturbance, however qualitative trends were observed in these data: zones of intermediate disturbance had peak parasite prevalence. Co-infections, however, were shown to operate independently, indicating a lack of ecological interaction between these particular parasites.

Introduction

Habitat disturbance and fragmentation can cause broad and sweeping effects to community composition and ecological interactions (Sih et al. 2000, Allan et al. 2003, McCallum and Dobson 2002, Patz et al 2004, Tabarelli et al. 2004); reducing overall species diversity, manipulating species abundance (Ferraz et al 2003, Laurance and Bierregaard 1997), and lowering animal health (Martínez-Mota 2007). Forest fragmentation, in particular, is generally recognized as one of the largest threats to global biodiversity (Fahrig 2003, Castro and Fernandez 2004).

Human activity and expansion that incurs land use change can result in habitat fragmentation and is on the rise due to an increasingly high demand by human populations for forest products (timber, bush meat) and agricultural development (Round-Turner 1994, Brooks et al. 1999, Peres 1990). The East Africa highland regions alone have experienced heavy deforestation, with 2.9 million hectares of forest cleared over a 9-year period (Food and Agriculture Organization 1993).

There is a growing body of literature to show that these changes also result in consequences to host-parasite interactions (Vaz et al. 2007, Cottontail et al. 2009, Gillespie and Chapman 2008). Habitat disturbance may further endanger wildlife populations as they raise parasite prevalence in communities. This raised prevalence results in increased numbers of interaction between wild and human populations, raising the possibility for novel parasite interactions or zoonoses (Chapman et al. 2005).

Studies in Uganda (Lindblade et al. 2000) and Tanzania (Bodker et al. 2003) have shown that land use change may be responsible for exacerbating human malaria epidemics, although other factors such as climate change (Loevinsohn 1994, Martens et al. 1999) and drug resistance (Bodker et al. 2003) may also be responsible. Habitat disturbance also impacts the many insect populations that serve as vectors for both human and wildlife diseases. Deforestation in Kenyan highland sites resulted in a 77.7%

increase in vector capacity for the transmission of human malaria, *Plasmodium falciparum* (Afrane et al. 2008). Changes to vector habitats influence biting, reproductive, and survival rates of vector species (Afrane et al. 2008, Vittor et al. 2006, Afrane et al. 2005, Rua et al. 2005, Afrane et al. 2006), indicating that habitat disturbance may have profound impact on vector-borne diseases.

Although many host-parasite studies on disturbed habitats have included surveys on multiple parasite species (Vaz et al. 2007, Cottontail et al. 2009, Gillespie and Chapman 2006), few have explicitly explored the within-host interactions between these parasites in relation to anthropogenic change. Natural host populations carry a diverse array of co-infecting parasites (Read & Taylor 2001, Pederson & Fenton 2006) and provide a natural laboratory for testing these interactions. It has been illustrated in both the laboratory and the field that co-infections define a whole range of within-host ecological interactions whose outcomes affect host and parasite evolution (Arnot 1998, Mercereau-Puijalon 1996, Daubersies et al. 1996, de Roode et al. 2005, Råberg et al. 2006, Barrow & Page 2000, Sernicola et al. 1999, Lipsitch 1999). Infection by one parasite may either facilitate or hinder the infection by another through a range of intraspecific or interspecific interactions (Chappell 1969, Ishii et al. 2002, Selva et al. 2009, Raberg et al. 2006, de Roode et al. 2005). It is these within-host interactions that occur on the backdrop of habitat disturbance in natural systems.

The present study aims to explore the effect habitat disturbance has on the prevalence of two genera of vector-borne haemoparasites of small mammals: *Plasmodium sp.* and *Trypanosoma sp.* I hypothesize that sites of disturbance will have higher relative prevalences of these parasite genera when compared to pristine locations. This research is interesting as the current body of literature has yet to explore if mosquito and Tsetse fly vector-borne diseases exhibit similar results to other vector disease systems over a range of disturbed habitats.

Rodent malaria (*Plasmodium sp.*) and *Trypanosoma sp.* parasites have both been used in the study of intraspecific competition (de Roode et al. 2005, Balmer et al. 2009). These parasites are ideally suited for studying within-host interactions as they both occupy similar host 'environments' (blood and tissues), result in generalized immunosuppression (Askonas and Bancroft 1984, Mendis et al. 1990, Riley E et al. 1988), and cause anemia by either erythrocyte lysis due to intracellular replication (*Plasmodium sp.)* or auto-antibody and compliment mediated erythrocyte lysis (*Trypanosome sp.)* (Rickman and Cox 1979, Amole et al. 1982, Li et al. 2001). These two have also been classically studied for interspecific interaction in laboratory mice (Cox 1975), where malaria infection (*P. yoelii)* was found to enhance secondary infection by trypanosomes (*T. musculi)*. Similar research found a reciprocal relationship for malaria infections (Hughes and Tatum 1956, Shmuel et al. 1975). The laboratory studies indicate that a positive interaction (co-exploitation of host) between these two genera is occurring (Mutualism).

Collecting information from these various studies, several different kinds of ecological interactions are possible between these genera (Table 2.1). Exploitation competition could arise if host nutrients, energy stores, or metabolites that are required by parasites become scarce. Whichever is most efficient at their acquisition would likely predominate in infections and potentially result in competitive exclusion. Exploitation competition is a possibility, especially when considered directionally from *Trypanosoma* to *Plasmodium* as trypanosome parasites are known to induce host anemia via erythrocyte lysis (Rickman and Cox 1979, Amole et al. 1982, Li et al. 2001). Since *Plasmodium sp.* require and inhabit erythrocytes in the mammalian host for asexual replication, this mechanism of anemia could directly impact their density. Such a competition could arise from needs for iron/heme or energy resources used by *Plasmodium* that would benefit *Trypanosome* infections if co-opted. Although there is

not direct adaptive immune-response crossover between *Trypanosoma* and *Plasmodium* infections (Cox 1972), generalized innate responses could still establish apparent competition between these genera. While both parasites would receive negative effects by such an interaction, one would benefit, relative to the other.

While interspecific laboratory evidence is indicative of a mutual exploitation of the host (mutualism), competitive interactions as described may be possible, as hosts from natural settings may be more restricted in their resources and health as compared to laboratory rodents, thus have open possibilities for competition to occur (Table 2.1). Analyzing the dataset for evidence of interactions will inform future studies if explicit exploration for interaction mechanisms are required. From prevalence data, estimates of single and co-infections will be made to test for interaction between parasites. Calculations will be made from the population as a whole and from particular sites of disturbance to see if habitat disturbance influences parasite interactivity. Since the laboratory models of these parasites illustrated interaction through increased parasitemia during co-infection, I anticipate finding a positive interaction between them in my dataset. Furthermore, I tentatively expect these two genera to exhibit a mutualism that will be observed by a positive trend between prevalence of one and infection status of the other. The benefit of co-infection will outweigh resource limitations, resulting in no discernable competition. If observed, this finding would represent the first field-collected data of within-host mutualism between *Plasmodium* and *Trypanosoma sp*. parasites.

Collections for this study accompany other research aiming to analyze small mammal diversity across disturbed habitats. As a result, the sample set will represent a broad range of small mammals (primarily rodents). Such a collection will give a good demographic snapshot of host and parasite species over the collection period. However, preliminary demographics will be looked at to determine if potential bias exists in our

sampling, as the potential for over-sampling species or parasites in only particular species is present.

The main goal of this study is to determine the prevalence of *Plasmodium sp*. and *Trypanosoma sp*. haemoparasites from a field collection of small mammals in an East African forest where no prior study of malaria or trypanosome infections of small rodents has been conducted. The dataset will be analyzed for 1) effect of habitat disturbance on parasite incidence and co-infection 2) signs of parasite interaction through a test of independence and comparison of prevalence by infection status 3) demographic bias from sampling hosts of potentially species specific parasites; in order to ask preliminary hypotheses about co-infection over a range of disturbed habitat and continued field analysis for rodent haemoparasites at this location.

Ecological Interaction	Mechanism	Resource	Evidence Required
Exploitation Competition	Resource acquisition, competitive exclusion	Energy, metabolites, oxygen, iron/heme	Established interaction between parasites, resource measurements
	Anemia-mediated-*Trypanosoma* lysis of *Plasmodium* infected cells	Iron/heme, host resources	Observation of anemia induction reducing *Plasmodium* population density
Apparent Competition	Immune-mediated	'Innate immune response freedom'	Negative relationship between parasite prevalence, described immune induction that is harmful
Mutualism	Immunosuppression, co-exploitation of host	Resources not limited or overlapping	Positive relationship between parasite prevalence (Cox 1975)

Table 2.1: Potential ecological interactions between *Plasmodium* and *Trypanosoma sp.* based on resource requirements and hypothesized interactions in natural host systems. Not all necessarily occur; investigation into particular lines of evidence will be explored if current study finds evidence for interaction in host populations.

Materials and Methods

Study sites and species

Blood samples were collected from small mammals in Kibale National Park and surrounding areas in western Uganda. Kibale is a 795-km^2 forested park near the foothills of the Rwenzori Mountains (0°13' -0°41'N, 30° 32' E) and represents a transitional region between lowland rainforest and montane forest (Figure 2.1). It consists of evergreen and moist semideciduous forest with intermittent grassland, woodland, lakes and wetland, colonizing forest, and plantations (Struhsaker 1997). These areas provide a patchwork of habitats that have undergone different levels of anthropogenic disturbance (logging, hunting, farming, village construction) that are optimal for the study of habitat disturbance and fragmentation. Sample collection sites (N=10), previously ranked on a range of least disturbed to most disturbed, include CC (pristine forest), K14 (lightly logged forest), K15 (heavily logged forest), forest edge, Bugembe (forest fragment 1), Kiko (forest fragment 2), the Makere University Biological Field Station (MUBFS), and homes and fields in surrounding villages (Kanyawara, Kaburara, and Ibura) [May – July 2009]. For analysis, the field station and surrounding villages were calculated together as they represent human occupied habitats and likely have similar small mammal populations. This is supported by the dataset, as the invasive species, *Rattus rattus,* almost exclusively belonged to human occupied locations with one exception.

All animal collections followed Institutional Animal Care and Use Committee (IACUC) protocol approved by Emory University (#062-2009) and the Centers for Disease Control and Prevention (CDC) (#1768).

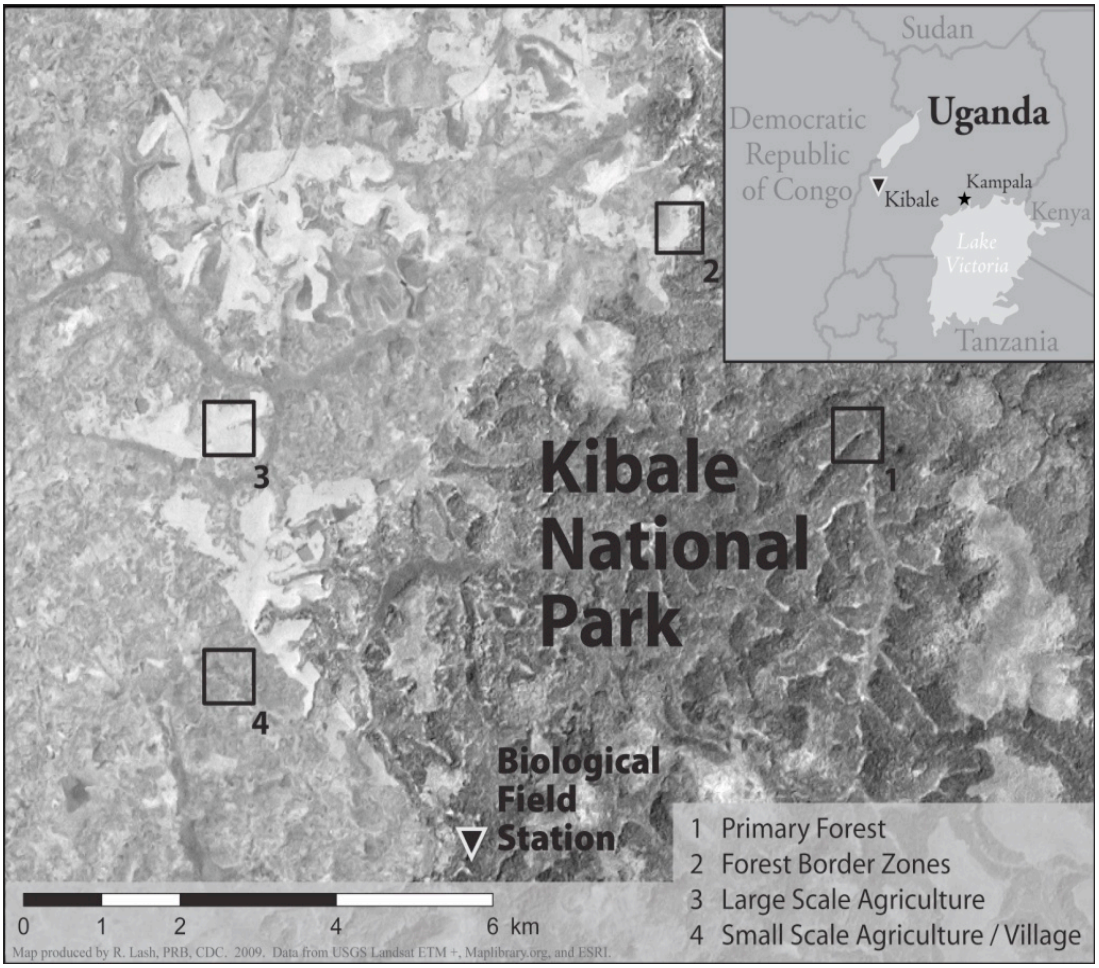

Figure 2.1: Arial satellite image of the northwestern edge of Kibale National Park located in western Uganda. Grayscale intensity correlates with vegetation and land cover type. The (1) primary forest is the darkest grey (2) forest edge includes areas of both primary forest and small scale agriculture, (3) large scale agriculture consist mainly of tea plantations and is depicted as the lightest grey color, and the (4) Villages and small scale agricultural plots are an intermediate shade of grey.

A field team sponsored by the Emory University Global Health Institute (GHI) collected all samples in this study. Animals were trapped at each site using 200 m diameter trapping webs (Paramenter et al. 1998, Mills et al. 1999).

Each web contained 148 trapping stations located along 12 transects subdivided by 12° from the web center. Both Sherman® and Tomahawk® model traps were used. This collection method was used to produce an accurate sampling of the rodent community at each site for later biodiversity estimation by the collecting team. Blood was collected intracardially while animals were under general anesthesia using Isoflurane. All animals were euthanized via overdose of anesthetic. Rodent genus and species identification is ongoing (Field Museum, Chicago) and is done using both morphometric and skull morphology analysis. Small mammals that have yet to have their identity confirmed, however, broadly belong to the Family Muridae, and likely contain *Praomys stella*, *Pr. jacksoni, Thamnomys dolichurus, and Th. rutilans* (Salzer, pers. Comm.), as these species are commonly found in Kibale, in the habitats sampled from, and are most similar to collected samples. All identified species represent the entirety of their respective clades.

Two thin blood smears were prepared for each animal and allowed to air dry completely before fixation. Smears were fixed in pure methanol for no more than one minute and dried again before storage in slide boxes with cotton, to prevent condensation. Of all slides collected (N=213), 199 were used in the microscopic inspection for haemoparasites. 14 slides were unusable or damaged due to improper fixation or thickness. These represent a total sample set of 103 small mammals.

Microscopic Analysis

Slides were stained in a 10% giemsa staining solution, prepared from a commercially available stock solution of giemsa, for 20 minutes. Each was flushed with water to remove excess stain upon removal from stain bath and allowed to air dry. Inspection of blood smears was done using standard light microscopy with phase contrast on an Olympus BX51 compound light microscope. Each slide was then visually inspected at low magnification (100x) to determine if blood smear on slide was sufficient for microscopic examination for haemoparasites. Presence of red blood cells (RBCs) over at least a 1cm x 1cm area of the slide was considered sufficient to warrant high magnification (1000x oil immersion) inspection. Approximately 100 fields of view were observed at high magnification for each slide. Counts were taken for infected RBCs to establish if *Plasmodium sp*. infection was ongoing at time of collection. Presence or absence notation was also made for *Trypanosoma sp*. concurrently by inspecting for trypomastigotes in the blood smear. Images were taken for illustration of representative parasite morphologies using an Olympus DP71 digital camera attachment and DPController software (Figure 2.2).

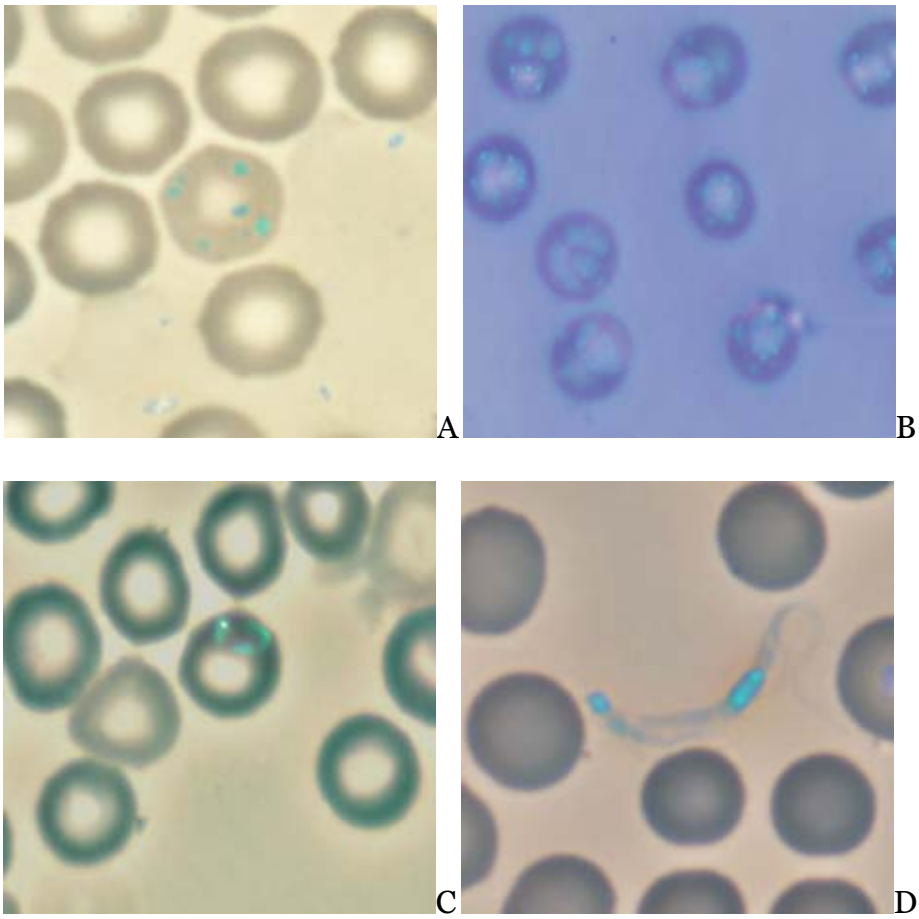

Figure 2.2: Microscopic images of haemoparasites. Images taken at high magnification (1000x oil immersion) under phase contrast. A) *Plasmodium sp*. schizont B) *Plasmodium sp*. schizont C) *Plasmodium sp*. ring D) *Trpanosoma sp*. trypomastigote.

Statistical Analysis

Statistical analyses were performed with R version 2.10.1 (R Foundation for Statistical Computing, www.r-project.org). Data were tested for demographic bias by host type with a chi-square (Yate's correction) 'goodness of fit' test. *Plasmodium sp. p*arasite prevalence data for disturbed habitats were tested with chi-square (Yate's, correction) and Fisher exact 2x2 contingency table tests individually against samples from the pristine forest, CC. Statistical testing was not performed on trypanosome data per habitat, as counts were considered too low for proper statistical comparison.

Another chi-square (Yate's correction) 'goodness of fit' test was applied to parasite prevalence by host type. Since full identification of host genera and species has not been completed at this time, this test analyzes for a broad definition of demographic bias. This could either be interpreted as too many (or few) hosts of particular type collected, or a different than normal infection prevalence in individual host types.

Coinfection data from the population was also analyzed. Parasite prevalence for each genus was plotted for presence and absence of the other parasite for comparison. Independence of haemoparasite infections was tested to determine presence of possible within-host interactions across the entire population sampled (Jolles et al. 2008). Calculation of expected number of co-infections ($E_{M<T}$) were calculated following Eqn. 2.1,

Eqn. 2.1: $E_{M<T} = Np_M p_T$

where N is the number of hosts and p_M and p_T are the prevalences of *Plasmodium sp. (M)* and *Trypanosoma sp. (T)* from the collected host population. The expected number

of single infections ($E_{M>T}$) of *Plasmodium sp. (M)* or *Trypanosoma sp. (T)* from the host population was then determined in Eqn. 2.2.

Eqn. 2.2: $E_{M>T} = (Np_M - E_{M<T}) + (Np_T - E_{M<T})$

These expected values represent the null hypothesis; that *Plasmodium* and *Trypanosoma* parasite infections operate independently of each other. Chi-square (Yate's correction) was used to determine if the collected values differed from the null expectation across all categories. This was then performed on individual habitats that experienced co-infections (K14, K15, Forest Edge, Human Occupied) to allow comparison of individuals within each habitat. This was done to determine if habitat disturbance influenced within-host interaction.

Results

Microscopic blood smear analysis of 103 individual small mammals resulted in prevalence data for *Plasmodium* and *Trypanosome* species. For all samples, 59.2% (N=61) were infected with haemoparasites. Of these, 48.5%(N=50) were infected with *Plasmodium sp.* while 17.5%(N=18) were infected with *Trypanosoma sp.* haemoparasites. These data were compared to haemoparasite prevalences of small mammals from the literature (Table 2.2). This analysis should be considered preliminary as larger sample sizes for each host and habitat type is desirable.

Haemoparasite prevalence by host showed large margins of error (Figure 2.2) due in part to heavy sampling from 3 genera or groups of rodents: *Rattus*, *Lophoromys*, and the 'unconfirmed' Muridae (Table 2.3). A 'goodness of fit' test was used to determine

if there was any demographic bias to sampling. Significant values were found for both genera of parasites χ^2 = 92.47 (df=8 p=2.2x10^{-16} for *Plasmodium sp.* and χ^2 =85 (df=8 p=4.8x10^{-15}) for *Trypanosoma sp.*. As these calculations included numerous 0 values, which may cause Pearson's χ^2 test to be incorrect, they were recalculated excluding these values which resulted in χ^2 =60.59 (df=6 p=3.4x10^{-11}) for *Plasmodium sp.* and χ^2 =16.33 (df=2 p=2.8x10^{-4}) for *Trypanosoma sp.*. Novel malaria infections were observed in several genera that, to my knowledge, have not been previously described as common hosts for *Plasmodium sp.: Lophuromys sp., Malacomys sp., Lemniscomys sp., and Tatera sp.*

The forest edge had the highest prevalence of *Plasmodium sp.* infections (75%) while K15 was only marginally higher than the forest edge for *Trypanosoma sp.* prevalence (38.5%) (Figure 2.4). K14 (33.3%) and the combined villages and station (32.0%) had the lowest prevalence of malaria. Bugembe (fragment 1) had the lowest *Trypanosoma* infection prevalence (13.3%) (Figure 2.4). χ^2 comparisons using 2x2 contingency tables of the pristine forest (CC) against disturbed habitats were all non-significant. Fisher's exact tests confirmed results from χ^2 analyses (Table 2.4).

Of infected animals investigated, 11.5% (N=7) had concomitant infections with both *Plasmodium* and *Trypanosoma* haemoparasites while 88.5% (N=54) had single infections (Table 2.5). For the whole sample set there was a negative trend between infection status of one parasite and prevalence of the other (Figure 2.5). Hosts infected with *Plasmodium sp.* had a 14% prevalence of concomitant infection with *Trypanosoma sp.* while hosts not infected with plasmodium parasites had a 20.8% prevalence of infection by trypanosomes (a 6.8% difference). Likewise, hosts infected with *Trypanosoma sp.* had a 38.9% prevalence of concomitant infection with *Plasmodium sp.* while hosts not infected with trypanosomes had a 50.6% prevalence of *Plasmodium* infection (an 11.7% difference).

In testing if co-infections of haemoparasites operated independently of each other (an indication of ecological interaction), no significant deviation from the null expectation was found in either the whole sample set or any meaningful subdivision by habitat type (Table 2.5).

	obs. Prevalence / total (%)	lit. prevalence / total (%)	Host (location)	source
Plasmodium sp.	50/103 (48.5)	6/41 (14.6)	*R* (Nigeria)	Killick-Kendrick (1968)
Trypanosoma sp.	18/103 (17.5)	7/138 (5.1)	R (Madagascar)	Laakkonen et al. (2003)
		43/276(15.6)	R (Thailand)	Jittapalapong et al. (2008)
		31/274(11.3)	M (Brazil)	Herrera et al. (2005)

Table 2.2: Number of observed haemoparasite infected small mammals as compared to previous studies from the literature of rodents infected with either *Plasmodium sp.* or *Trypanosoma sp.* R= comprehensive collection of Order Rodentia mammals. M= collection from various mammalian orders

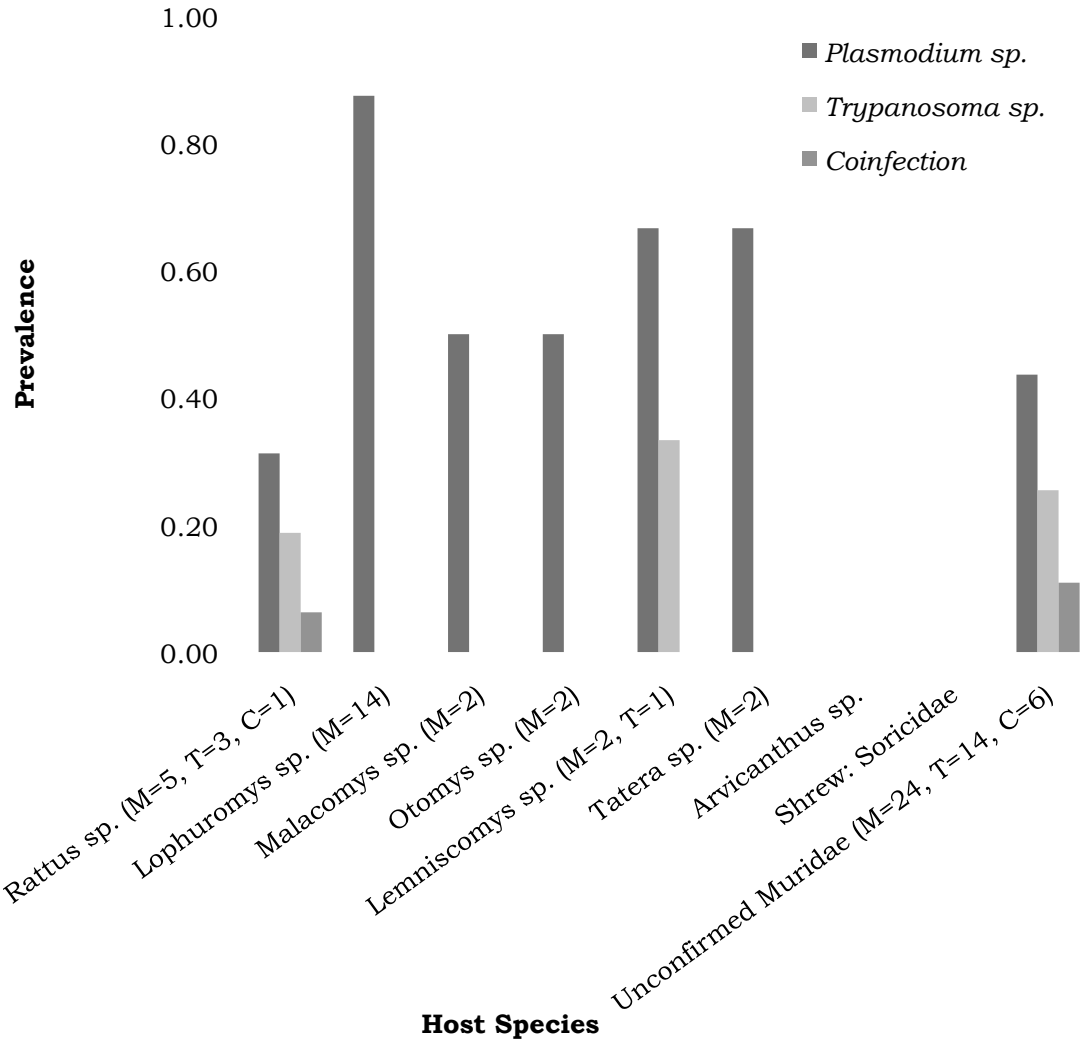

Figure 2.3: Prevalence of haemoparasites by host genus. dark gray= *Plasmodium sp.* ; light gray= *Trypanosoma sp.*; medium grey= co-infection with both plasmodium and trypanosome parasite. Uncapped error bars= Standard Error of Proportion (SEP) of the proportion infected of the total collected for each host genus. M= *Plasmodium* # infected, T= *Trypanosoma* # infected, C = # co-infections. The unconfirmed Muridae represent samples that have yet to have their identity confirmed by likely belong to Muridae genera: *Praomys* and *Thamnomys* (Salzer pers. comm.).

Host Genus	***Plasmodium sp. infection (%)***	***Trypanosoma sp. infection (%)***	**Co-infections (%)**	**Total**
Rattus sp.	5 (31.3)	3 (18.8)	1 (6.3)	16
Lophuromys sp.	14 (87.5)	-	-	16
Malacomys sp.	2 (50)	-	-	4
Otomys sp.	2 (50)	-	-	4
Lemniscomys sp.	2 (66.7)	1 (33.3)	-	3
Tatera sp.	2 (66.7)	-	-	3
Arvicanthus sp.	-	-	-	1
Shrew: Soricidae	-	-	-	1
Unconfirmed Muridae	24 (43.6)	14 (25.5)	6 (10.9)	55

Table 2.3: Infection prevalence by host genus. – marks represent no infections found in associated genus. All genera belong to family Order Rodentia.

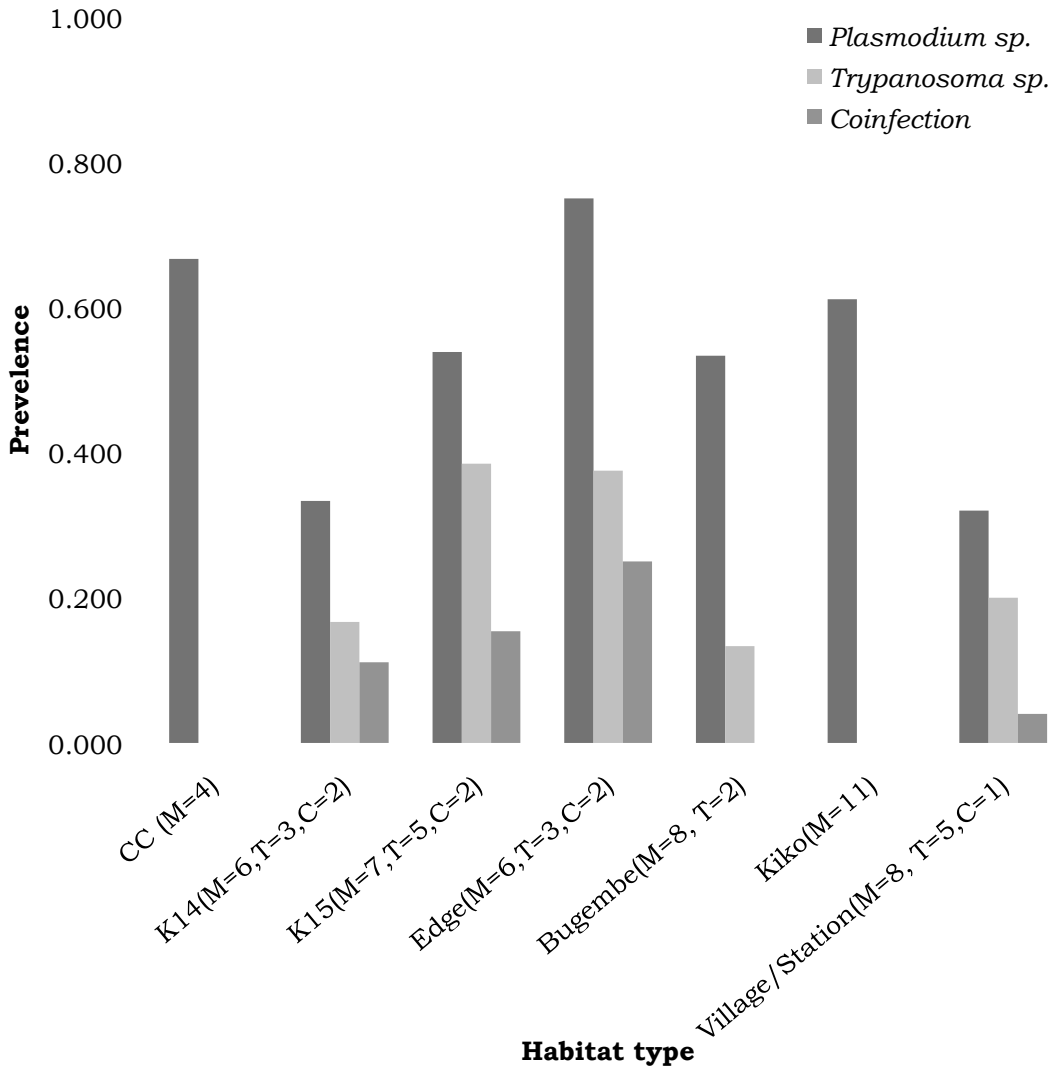

Figure 2.4: Haemoparasite prevalence by habitat type. Habitat disturbance is graphed linearly from low-pristine forests (CC) to high-heavily disturbed (Kiko and Human occupied villages and field station) dark gray= *Plasmodium sp.*; light gray= *Trypanosoma sp.*; medium grey= co-infection with both *Plasmodium* and *Trypanosoma* parasites. Error bars= Standard Error of Proportion (SEP) of the proportion infected with *Plasmodium sp.*, *Trypanosoma sp.*, or co-infected against the total of each habitat type. M= # *Plasmodium,* T= # *Trypanosoma*, C= # co-infected.

Habitat	*Plasmodium sp. (%)*	*Trypanosoma sp. (%)*	Total	2x2 Comparison of *Plasmodium sp.* infection against CC		
				χ^2	p	Fisher
CC	4(66.7)	-	6	x	x	x
K14	6(33.3)	3 (16.7)	18	0.914	0.339	0.192
K15	7 (53.8)	5 (38.5)	13	0.029	0.864	1
Edge	6 (75.0)	3 (37.7)	8	0.066	0.798	1
Bugembe	8 (53.3)	2 (13.3)	15	0.005	0.944	0.659
Kiko	11 (61.1)	-	18	0.001	0.971	1
Villages/ Station	8 (32.0)	5 (20)	25	1.21	0.272	0.174

Table 2.4: Haemoparasite incidence by habitat type. – mark represents no observed parasites. No significant values were found in 2x2 contingency analysis of disturbed habitats against pristine forest (CC).

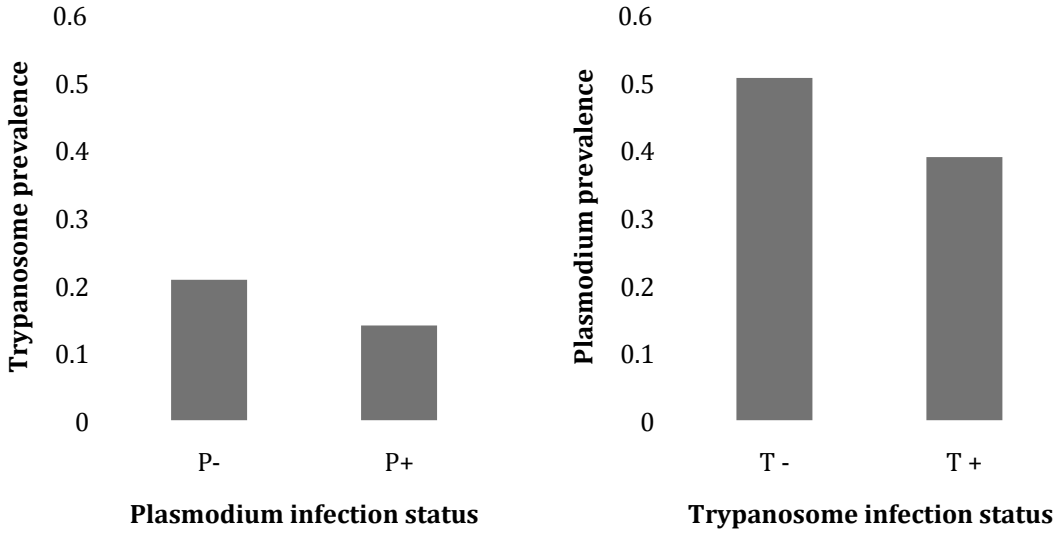

Figure 2.5: Correlations between infection status and infection prevalence between haemoparasites plasmodium and trypanosome. Graphs illustrate prevalence of alternate parasite based on infection status of its counterpart.

	Single infect. obs.	Co-infect. obs.	Single infect. exp.	Co-infect. exp.	χ^2 Single infect.	p Single infect.	χ^2 Co-infect.	p Co-infect.
Entire Sample	54	7	50.5	8.7	0.117	0.732	0.184	0.668
K14	5	2	7	1	0.333	0.564	0.333	0.564
K15	8	2	6.6	2.7	0.134	0.714	0.104	0.7468
Edge	5	2	4.5	2.3	0.026	0.871	0.021	0.885
V/S	11	1	9.8	1.6	0.069	0.793	0.139	0.710

Table 2.5: Co-infection of haemoparasites by habitat type; calculation for independent of interaction from expectation values. No significant interaction was found for any co-infection data.

Discussion

Statistical analysis of the dataset failed to produce any significant interaction between haemoparasite prevalence and host habitat type (Table 2.4) when comparing between pristine forest and disturbed habitat. Problems arose in making 2x2 comparisons as the sample size for pristine forest (n=6) was sufficiently low to warrant the use of Fisher exact tests; χ^2 analyses become unreliable at low data counts.

Visual inspection of these data, however, reveals possible trends in parasite prevalence to host-habitat type (Figure 2.4). Ignoring the pristine forest sample (CC), due to low sample size, haemoparasite prevalence appears to rise to a peak at levels of intermediate disturbance, along the forest edge, before lowering again at high levels of disturbance, areas continually occupied by humans. Caution must be observed when evaluating these data as such, as other confounding variables may influence parasite prevalence. It is to note that both forest fragments sampled from, Bugembe and Kiko, were noticeably wetter than any other fragment sampled from (mud and runoff streams from higher elevations). This increase of moisture could potentially influence the vector composition of these areas, and likewise, any parasites they transmit.

Low parasite prevalence in lightly disturbed habitat is not uncommon in other host-parasite systems. Gillespie et al. (2010) illustrated that habitats with historical low levels of disturbance may exhibit baseline gastrointestinal parasite prevalence that does not differ from undisturbed habitats. If applicable to the present study system, samples collected from the lightly logged forest (K14) plot in Kibale may be representative of parasite prevalence expected in undisturbed forest plots. As a result the small sample collected from the pristine forest may be an outlier. A more robust analysis of a larger dataset will be necessary to elucidate this. At present, I am unable to conclude if habitat disturbance significantly influences small mammal haemoparasite prevalence in Kibale.

While the data presented suggest that haemoparasite prevalence was highest for *Plasmodium* infections and second highest for *Trypanosome* infections in the forest edge habitat, these transitional areas have previously been considered less conducive to parasite transmission. Since forest fragmentation and disturbance may change the microclimate of host-habitats (Murcia 1995), the forest edge in particular may be less hospitable to parasite transmission (Gillespie and Chapman 2008) as it has increased wind, solar radiation, and is drier than the forest interior (Fetcher et al. 1985, Murcia 1995). This certainly applies to gastrointestinal parasite systems, where disease transmission is facilitated through the fecal contamination of habitats and parasites are more readily exposed to the elements (Larsen and Roepstorff 1999), however, vector-borne parasite systems, with no external stages, may not be as prone to direct environmental forces.

Previously, deforestation was shown to increase vector capacity for parasite transmission (Afrane et al. 2008). Increased vector capacity was strongly attributed to higher temperature microclimate changes that are also observed along forest edges. Also important for disease transmission in vector systems is the encounter rate (number of blood meals taken) between vector and host. This will be maximized in the habitat that provides the most suitable resources (food, water, nesting sites) and environment (survivability, reproduction) for both vector and host. A maximized habitat will exhibit the highest prevalence of vector-borne infections. Future studies should investigate the encounter rate of vectors and hosts to better define this relationship to habitat disturbance with special consideration to the forest edge.

If these data trends hold true, lower infection prevalence in human occupied areas may be explained by the use of human disease prevention devices such as nets and insecticides that lower the overall prevalence of vectors. An alternative explanation for reduced prevalence is that human settlements have different communities of hosts and

vectors that offer alternative ecological interactions at the host, vector, and parasite level. If this is true, the current study would be unable to differentiate its lack of results from higher scale ecological interactions, as the host identity database is incomplete and unable as of yet to determine definitively which hosts live where. Furthermore, the current study did not differentiate between possible mixed infections within individual hosts. Advanced microscopy skills or molecular work is required for identification beyond the genus level for *Plasmodium* and *Trypanosom*a species. Future work needs to address these issues, and explore the possibility of other parasite interactions within the host that may prevent or enhance concomitant infections.

The opposite of my hypothesis occurred for co-infected hosts; a negative trend was observed between parasite prevalence and infection status for either *Plasmodium* or *Trypanosoma* infected hosts (Figure 2.5) suggesting that a mutualism is unlikely between these parasite genera. Analysis of concomitant infections showed no significant interaction between *Plasmodium* and *Trypanosoma sp*. (Table 2. 5). Both of these findings are surprising in contrast to the classical laboratory studies (Cox 1975), where *Plasmodium* positive hosts had increased parasitemia of *Trypanosoma* infections. This result could be illustrative of the key differences between laboratory and naturally infected hosts and how they respond to infection. Rodents in laboratory studies lie in stark contrast to natural hosts for several reasons. Primarily, they are not the natural hosts as are observed in field collection and may have entirely different immune capacity for dealing with single and co-infection by haemoparasite infection. Laboratory rodents are also not placed under natural dietary or health limitations that is observed in natural populations. These controlled conditions likely skew the host's nutritional resources and health to the high end of the spectrum while field-collected hosts could easily suffer from malnutrition, injury, or other such deficiencies. It is entirely possible that natural systems will carry a larger array of underlying ecological interactions as a result of this

heterogeneity in host resources and health, permitting the observation of such interactions like competition that would otherwise not be seen (Table 2.1).

While the current statistical analyses did not indicate any interaction was occurring between these parasite genera, this may be a by-product of small sampling size or ecological dynamics confounding the dataset from the host, vector, or parasite level. The current study did not take into consideration the density of hosts in particular habitats and how this crowding could influence parasite prevalence. Since this is a vector system, direct density-dependent transmission is not considered as the main mechanism for disease occurrence. Rather, Ross models, or R_o (basic reproductive rate) models that describe the number of secondary infections that result from a primary one may be used to incorporate density of host and vector (Zavaleta and Rossignol 2004). Such models incorporate density through a measure of vector-capacity that accounts for both the biting rate of vectors and the density of hosts to be bitten. Similar data can be collected in the field by determining the encounter rate between vectors and hosts. This may be important in evaluating such field systems as R_o can serve as a description of parasite persistence in a population ($R_o>1$: parasite persists in a population, $R_o<1$:parasite fades out of population). By establishing R_o and other indicators of disease interaction over a population of organisms, future studies will benefit in building consensus on how within-host mechanisms interact with host-population level infection studies.

It is possible that there are other underlying layers of interaction in this natural system that would not be observed in laboratory. Time between primary and secondary infection, host compatibility, multiple inoculations, and parasite establishment within the host could all be important determinants of how within-host interactions occur.

Parasite prevalence significantly differed from expected when samples were grouped by host type (Table 2.3). Although the intention of this analysis was to determine if there was demographic bias in our sampling, it could also indicate that

particular small mammal species are more prone to haemoparasite infection. Many *Plasmodium sp*. positive samples belonged to the unconfirmed Muridae grouping. While the potential genera these rodents belong to has been narrowed down based on species prevalence in Kibale National park and gross morphology comparison (Struhsaker 1997, Salzer pers. comm.), these animals necessarily need to be identified before deeper analysis of their habitat distribution and parasite prevalence can be undertaken. Furthermore, without deeper investigation into the parasite species infecting hosts, to determine if any are species specific and not generalists, no reasonable conclusions can be made on haemoparasite incidence over the small mammal community in Kibale National Park. Future work on the larger molecular dataset that accompanies this study will need to analyze phylogenetic data of both host and parasite as well as ask questions about their co-evolution.

Interestingly, novel *Plasmodium sp*. infections were detected in several small mammal genera that have not, to my knowledge, previously been observed with *Plasmodium* parasites: *Lophuromys sp., Malacomys sp., Lemniscomys sp., and Tatera sp*. Common hosts of rodent malaria parasites that were not identified are likely members of the 'unconfirmed Muridae' grouping (Salzer pers. comm.). These novel infections need to be verified by both full identification of host species and molecular analysis of parasite presence to further describe their incidence. Their presence, however, provides the interesting possibility of cross species infections due to the legacy of habitat fragmentation and disturbance at Kibale.

Although this preliminary study falters on making firm statistical inferences on haemoparasite prevalence and co-infection over a range of habitat disturbance, it is able to make critical observations from qualitative analyses. Kibale appears to be an ideal location for future investigation of small mammal haemoparasites as prevalence across genera investigated was relatively high as compared to past research (Table 2.1).

Reinvestigation of Kibale's undisturbed forest (CC) for more data will be necessary to make headway into resolving suspected trends from habitat prevalence and much needs to be elucidated regarding parasite and host species collected.

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3) Study of habitat disturbance and fragmentation; effects on within-host parasite ecology: Future directions

Preliminary data presented in Chapter 2 illustrate many of the pitfalls of working with field-collection systems. Small samples sizes prevent any real analysis without making drastic assumptions or exceptions that are not supported by rigorous hypothesis driven research. Fortunately, these data presented are just preliminary with a three-times larger set of dried blood filter paper slides and spleen samples that were collected concurrently and are waiting to be analyzed. Future analyses of this expanded dataset by both molecular and antibody detection methods will verify the current microscopic collection and add much needed sample size to this study.

As was indicated, a visual inspection of these data revealed a possible trend in parasite prevalence to host-habitat type that peaked at areas of intermediate disturbance. Whether this trend is spurious or not will be clarified by later analysis. It does however raise interesting questions about how the spectrum of habitat disturbance interacts with host-parasite systems. Two bodies of literature can be used to understand this observation: the intermediate disturbance hypothesis and the edge effect.

The intermediate disturbance hypothesis states that species diversity should be maximized when disturbance is balanced at an intermediate level (Connell 1978). At this intermediate level, the number of species lost as compared to the number of species gained will be high. If these processes are true, habitat invasion may be a likely outcome (Hobbs and Huenneke 1992). For host-parasite systems, I would expect this to be realized as an increase in abundance of generalized hosts, vectors, and parasites that are able to take advantage of a varied environment. Applying these principles to haemoparasites of small African mammals, I would anticipate an abundance of invasive

or high tolerant mammals (and their associated vectors) to occupy areas of intermediate disturbance. Overlap in species distribution between high and low disturbed habitats would be expected in these intermediate ranges. This increased interaction between natural species, and likely, the humans propagating anthropogenic disturbance, opens opportunity for interaction and recombination of once isolated parasite species, resulting in novel zoonoses.

These interactions, however, may be confounded when compared across large groups of organisms. Interactions at the genus level, may not be descriptive of true within-host ecological interactions as individual species will vary in their host specificity, infection cycles, and necessary resources. While the overall diversity at locations of intermediate disturbance may be maximized, and this may be apparent at higher levels of classification (such as genus), this does not necessarily mean parasites will have the means of interacting across diverse groups of host organisms. How pliable particular pathogens are at using a diverse range of hosts will be important in governing how often new parasite interactions will occur at these interface regions. Deeper resolution into parasite and host species should provide more representative analysis of the 'true' biological processes that underlie the system.

How disturbance effects species within a habitat is determined both by how a species responds to a disturbance and how other species that interact with that particular species respond to a disturbance (Hobbs and Huenneke 1992). Cascading effects like these are expected to be common in complex host-parasite systems, making them a suitable study system for exploring these questions.

Habitat fragmentation is a level of disturbance that covers a large range, resulting in the development of new habitat edges. For forest systems in particular, the edge effect can have negative consequences on forest maintenance (Murcia 1995). These negative consequences may be attributed to increased invasion and interaction of non-native

species with those native species remaining in habitat fragments. While previous considerations have indicated that the forest edge is not as conducive to some parasite transmission (Gillespie and Chapman 2008) due to altered microclimate effects such as increased wind, solar radiation, and lower relative humidity (Fetcher et al. 1985, Murcia 1995), this may not be the case for all parasite systems. While many vector systems are still prone to environmental factors such as temperature and humidity, past research has indicated that deforestation (the creation of forest edges) can increase the capacity of vectors by 77% (Afrane et al. 2008). Consideration of how all members in complex host-parasite interactions, including vectors, respond to habitat disturbance must be taken into account. Continual investigation into vector-borne haemoparasites necessarily will need entomological data to accompany host prevalence.

Prior to any recommended increase in field collection efforts, concrete evidence needs to be presented that illustrates interaction between parasites within any particular host. As was previously discussed, it is surprising that no interaction was detected between plasmodium and trypanosome haemoparasites. The combination of this result with the negative trend of parasite prevalence to concomitant infection raises questions as to which is a better descriptor of parasite dynamics across small mammal species in Kibale. This result also brings to question using results from laboratory studies to develop hypotheses for natural systems. The divergence between non-native hosts and controlled laboratory conditions from natural systems may make too many assumptions on how basic host-parasite interactions operate. Ecological dynamics that are controlled for in the laboratory (nutrition, stress, immune competency) are taken for granted when trying to compare to natural hosts.

Demographic bias in host or parasite observance may also have produced non-significant results and conflicting trends. Since microscopic detection methods employed only detected parasites at the genus level, multiple species or strains within each genus

could have been within single or multiple hosts. How these particular organisms compete with each other may have variation. Laboratory analyses of genetically diverse infections have shown that intraspecific competitive interactions can, and often arise (de Roode et al. 2005, Balmer et al 2009). This indicates that future research should heavily invest in molecular tools that can differentiate species and strains of parasites to a useful level of comparison.

Fortunately, tools for such goals are widely available. Genomes for both human and rodent malaria (Gardner et al. 2002, Carlton et al. 2002) and *Trypanosoma brucei* have recently been published (Berriman et al. 2005). With these resources on hand, developing robust methods for phylogenetic analysis that use functional genes on which evolution would operate is possible (Perkins et al. 2007). Using such an application will be beneficial in determining what mechanisms within a host are under selection. With these data, complex co-evolutionary relationships between host and parasite may be established, enabling the study of how habitat disturbance has affected hosts and parasites over evolutionary time. Any future research into the within-host ecology of natural systems should look to these technologies for resources that can enable both rapid and fine-scale analysis of samples collected.

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