CAUSES AND CONSEQUENCES OF COINFECTION: INSIGHTS FROM COMMUNITY ECOLOGY

by

SARAH ASHCOM BUDISCHAK

(Under the Direction of Vanessa Ezenwa)

ABSTRACT

Most animals are concurrently infected with multiple parasite species, and interactions among co-occurring parasites can influence disease dynamics and host fitness. I used a cross-sectional field study, a parasite removal experiment, a laboratory experiment, and a longitudinal field study to investigate the causes and consequences of parasite community composition. The four-year field study presented a unique opportunity to investigate parasite communities in a wild population of African buffalo (*Syncerus caffer*) with repeated sampling of condition, immune function, and coinfection status by multiple parasitic worms. My goals were to understand the drivers of individual variation in infection and to measure the costs of coinfection for hosts and parasites. To test the utility of a community ecology trophic framework for studying of parasite coinfection, I conducted a factorial experiment using laboratory mice as 'mesocosms' where two helminths (*Nippostrongylus brasiliensis* and *Heligmosomoides bakeri*) and a microparasite (*Mycobacterium bovis*) could interact. Resources altered the strength of interactions between coinfecting parasites and had opposing effects on the two helminths. Immune-mediated effects of coinfection were stronger than resource-mediated effects on parasite reproduction. Importantly, I documented stronger immune-mediated facilitation between micro- and macroparasites during

resource limitation that may have implications for susceptibility to and severity of major human pathogens because resource scarcity and helminth infection frequently co-occur. Examining the costs of coinfection in wild African buffalo populations, I found that one helminth species may decrease host survival via impacts on host condition, while, surprisingly, a closely related species was positively associated with host condition. This study is one of the first to empirically link coinfection in a wild population to changes in condition and survival. Together, the field and laboratory components of my dissertation highlight how different types of parasites interact with each other and with their hosts, and underscore the context-dependency of these interactions. More broadly, my research highlights that measuring the intermediate mechanisms by which parasites and hosts interact, namely via resources and immune function, can provide insight into the variability in coinfection outcomes for hosts and parasites.

INDEX WORDS: wildlife disease, parasite infection, helminth, African buffalo, ecoimmunology

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In loving memory of Timothy Rion Wade, II

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

Identifying factors influencing parasite transmission and the costs of infection for individuals and populations is a long-standing, central goal in disease ecology. Historical approaches addressing this goal are typified by single-species infection studies and cross-sectional surveys (reviewed in (Bordes and Morand 2011). However, correlational data from cross-sectional approaches cannot be used to infer directionality of most host-parasite interactions. For example, field studies often detect high infection levels in poor condition hosts, but it is unclear if poor condition led to increased parasite susceptibility (via decreased immune function), if parasite infection decreased host condition directly, or if both processes work synergistically in a "vicious cycle" (Beldomenico and Begon 2010). Similarly, while the emerging field of eco-immunology is expanding techniques for measuring immune function in wild hosts, single time point measures cannot be used to understand the fitness costs and benefits of different immune defense strategies (Graham et al. 2011). In lieu of correlative studies, longitudinal and mechanistic approaches are needed to improve our understanding of the outcomes of host-parasite interactions for parasite transmission and host fitness.

The presence of multiple parasite types in individual hosts and within host populations has long been acknowledged (Petney and Andrews 1998), but the ecological and evolutionary repercussions of coinfection for hosts and parasites has only recently been demonstrated in a handful of studies (reviewed in (Bordes and Morand 2011). Compelling, but limited, evidence

from studies taking a multi-parasite perspective suggest that interactions among co-occurring parasites can influence both disease dynamics and host fitness in the wild (Lello et al. 2004, Marzal et al. 2008, Ezenwa et al. 2010, Telfer et al. 2010). For example, a recent longitudinal study of voles showed that host infection status with three parasites explained more of the variance in disease risk across individuals than factors such as sex, season, and condition, traditionally assumed to be key determinants of susceptibility (Telfer et al. 2010). Importantly, such individual-level effects may scale up to shape both host and parasite population and community dynamics. These broader impacts of coinfection are exemplified by the recent global decline in honeybees due to colony collapse disorder (CCD), where the co-occurrence of a DNA virus and a fungal pathogen best fit the pattern of hives affected by CCD, and mortality was accelerated in bees experimentally exposed to both parasites (Bromenshenk et al. 2010). Despite the frequency, diversity, and potential impacts of multi-parasite infections in wild hosts, the factors that shape parasite community composition and the consequences of parasite community composition for host and parasite fitness remain a critical knowledge gap (Bordes and Morand 2011, Graham et al. 2011).

For my dissertation research, I used a multifaceted approach that combined the strengths of a cross-sectional field study (Ch.1), a parasite removal field experiment (Ch. 2), a laboratory experiment to manipulate infections and host resources (Ch. 3), and longitudinal field study (Ch.4) to investigate the causes and consequences of parasite community composition. My research focuses on helminth coinfection and interactions between helminth communities and microparasites (e.g. bacteria and protozoa). Helminths infect over a third of the human population and are nearly ubiquitous in wild animal populations (Cox 2001, WHO 2010). Immune defenses to helminths and microparasites are mutually inhibitory, and may create

indirect, positive interactions (i.e. the enemy of my enemy is my friend) (Cox 2001). Consequently, helminth infection is known to affect susceptibility, virulence, and transmission of important human and wildlife microparasitic diseases (e.g. HIV, malaria, bovine tuberculosis, cowpox) (Ezenwa and Jolles 2011, Lustigman et al. 2012, Salgame et al. 2013).

In Chapter 2, I examined the relationships between multiple concurrent GI parasite infections and host hematological profiles and body condition in free-ranging African buffalo (*Syncerus caffer*). I used a combination of morphology-based aggregate parasite data and molecular-based species-level data to examine the relationship between parasite richness and abundance on four distinct host hematological parameters as a proxy for host condition. Since immune defenses are energetically costly to develop and maintain, and may indirectly affect host physiology and performance, I also tested for relationships between immune investment and red blood cell parameters. Finally, using species-specific parasite data, I examined the degree to which species-specific and aggregate parasite abundance correlated with condition measures and immune investment. This cross-sectional study was used to generate specific hypotheses for subsequent longitudinal studies and experiments.

To better understand the role that interspecific interactions play in shaping parasite community composition, I used an anthelminthic treatment-induced disturbance to examine the assembly processes that shape parasite community structure in wild African buffalo. In chapter 3, I tested for effects of disturbance on the relative abundance of individual helminth species, and then examined impacts on four measures of community structure: total abundance, richness, diversity, and evenness. I also explored recolonization patterns by evaluating the relationship between time since disturbance and community structure in treated hosts. Importantly, in this

chapter I explored whether similar inferences about parasite community composition could be drawn using the non-lethal parasite sampling techniques.

Coinfection outcomes depend upon both the identity of the parasites involved and the mechanisms by which they interact. Accordingly, not all helminths have equivalent effects on microparasite susceptibility and host fitness (Pedersen et al. 2007, Graham 2008). It has been suggested that the ecological trophic framework can be adapted to explain variability in coinfection outcomes (Pedersen and Fenton 2007). When a traditional community ecology trophic framework is applied to parasites, "attacks" by the host's immune system are analogous to top-down, predation pressure, while host resources exert bottom-up effects on parasites by limiting critical nutrients (Pedersen and Fenton 2007, Graham 2008). Notably, additional indirect interactions between hosts and parasites arise because the top and bottom trophic levels are directly linked because host immune responses often depend of resource availability (French et al. 2009). Although an insightful meta-analysis demonstrated that this trophic framework shows great promise for understanding micro- and macroparasite coinfections (Graham 2008), its utility had not been previously tested empirically. To investigate how resources and immunity influence interactions among co-occurring parasites as well as host and parasite fitness, I conducted a factorial coinfection experiment using a laboratory mouse model with two resource treatments, two helminths that trigger different immune responses, and a microparasite infection challenge (Chapter 4). To understand the complex network of interactions in the parasite trophic framework, I combined structural equation models with more traditional analyses to quantify the direction and strength of connections among parasites, resources, and immunity.

For chapter 5, I explored links between helminth infection and components of host physiology and fitness. I used a 4-year longitudinal study of the gastrointestinal parasites of

African buffalo. Using a novel molecular approach to distinguish helminth species shed in the feces and quantify helminth communities within live hosts, I examined how changes in the abundances of individual helminth species were associated with changes in host condition, immune function, microparasite infection, reproduction, and survival. While experimental studies are a gold standard for discerning causal effects of parasites on host fitness, the inability to selectively manipulate individual members of the helminth community make it difficult to use this approach for understanding the effects of individual helminth species and community composition on host fitness. Previous cross-sectional work in African buffalo species (Ch. 1) revealed that the two most common helminth species in the community may have opposing effects on physiological indicators of host performance and immune function, and that effects of the more pathogenic species (*Haemonchus* sp. [H]) were masked when considered in combination with the more abundant species (*Cooperia fulleborni* [Cf]) (Budischak et al. 2012).

Following up on these intriguing physiological patterns, I investigated the how changes in parasite infection were associated with changes in host condition, immunity, reproduction, and survival through time in 249 hosts recaptured approximately every six months. This longitudinal approach evaluated whether increases or decreases in the abundance of specific worm species corresponded to simultaneous positive or negative changes in host traits. Although this approach is correlational, it enables detection of species-specific effects potentially masked during experimental removal studies and provides stronger causal inference than single time-point studies. Using these dynamic patterns and previous experimental and cross-sectional studies of buffalo, I examined how each helminth species might directly and indirectly impact host physiology and fitness.

Collectively, this work aimed to determine if the complex interactions among hosts and parasites could be understood using mechanistic, species-specific, and longitudinal approaches. My research combines ideas from community ecology and ecoimmunology to measure the factors shaping parasite community composition and the consequences of infection for host and parasite fitness. Most organisms are coinfected with multiple parasites and live in environments where resource availability varies both spatially and temporally, so it is critical to understand the costs of parasite infection in these real-world contexts.

CHAPTER 2

DIRECT AND INDIRECT COSTS OF CO-INFECTION IN THE WILD: LINKING GASTROINTESTINAL PARASITE COMMUNITIES, HOST HEMATOLOGY, AND IMMUNE FUNCTION [1]

[1] Budischak, S. A., A. E. Jolles, and V. O. Ezenwa. 2012. Direct and indirect costs of co-infection in the wild: Linking gastrointestinal parasite communities, host hematology, and immune function. International Journal for Parasitology: Parasites and Wildlife. 1: 2-12. http://dx.doi.org/10.1016/j.ijppaw.2012.10.001.

Abstract

Most animals are concurrently infected with multiple parasites, and interactions among these parasites may influence both disease dynamics and host fitness. However, the sublethal costs of parasite infections are difficult to measure and the effects of concomitant infections with multiple parasite species on individual physiology and fitness are poorly described for wild hosts. To understand the direct and indirect physiological costs of co-infection, we investigated relationships among gastrointestinal parasite richness, species identity, and abundance and host hematological parameters, body condition, and investment in lymphocyte defenses. Using aggregate-scale parasite data from African buffalo (*Syncerus caffer*), we found few direct or indirect associations between infection and hematology in male hosts, and no significant associations were observed in female hosts or with respect to body condition in either sex. These results suggest that only strong physiological effects are detectable with aggregate-scale parasite data, and that hematological variables may be more sensitive to changes in condition than standard body fat condition indices. However, analyses accounting for parasite species identity in female buffalo revealed that different parasites show distinct relationships with host hematology, body condition, and immune investment. Furthermore, four of six species-specific associations were obscured when parasites were considered in combination. Overall, our findings suggest that the physiological costs of infection may be difficult to detect when co-infecting species are considered in combination. However fitness-related physiological mediators such as hematological indices may provide assessments of direct and indirect effects of parasite infection, particularly when parasite species identity and community composition are considered.

Introduction

Both microparasites (e.g. bacteria, viruses) and macroparasites (e.g. helminths, arthropods) can have far-reaching effects on the fitness of their hosts, ranging from reducing body condition below levels critical for reproduction to causing castration or direct mortality (Berger et al. 1998, Stien et al. 2002, Lafferty and Kuris 2009). The effects of parasites on host population dynamics have also been documented under a range of conditions and in an increasing number of host taxa (Tompkins and Begon 1999, Tompkins et al. 2010). Historically, most studies describing parasite effects on individual hosts or host populations focused on single parasite species, despite the fact that an overwhelming majority of hosts are simultaneously infected with multiple parasites (Petney and Andrews, 1998). More recently, however, both the number and specific identity of co-infecting parasites have been implicated as potentially critical determinants of the relative impact of parasites on hosts (Behnke et al. 2005, Craig et al. 2008, Telfer et al. 2010). These studies highlight the need for additional work focused on co-infection as it relates to host fitness and population dynamics.

Even for single parasite infections, the fitness costs of infection can be difficult to quantify in the wild. For example, in short-term or cross-sectional wildlife studies, host survival is rarely quantified, infection duration is unknown, and reproduction is typically calculated in terms of offspring produced during a single breeding period. Accordingly, estimates of parasite effects on host fitness are often difficult to determine. Longitudinal and experimental studies can provide accurate information on the costs of parasite infection when effects of parasites on host survival and/or reproduction are monitored over time (e.g. (Hudson et al. 1998, Stien et al. 2002, Telfer et al. 2008). However, the logistical difficulty and expense of these studies mean that comparatively few such studies can realistically be conducted in wild systems. When

longitudinal studies or experiments are not feasible, another alternative may be for short-term studies to use physiological performance indices as a currency for inferring the downstream costs of parasitism to the host.

Physiological indices (e.g. hormones, immunity, energetics) are intrinsically connected to performance and reproductive success (Moore and Hopkins 2009). Ecologists often measure energetic reserves by assessing stored fat, mass, or mass/size ratios as a measure of individual body condition. Alternatively, hematological profiles, including measures of the size and abundance of red blood cells and counts of white blood cell types, respond quickly to physiological perturbations (i.e. diet, stress, hydration status), and are widely used by veterinarians and physicians as indices of condition (Jain 1993, Yochem et al. 2008). Although rarely used by ecologists, hematological measures may provide additional or potentially more sensitive information on the physiological status of the host.

Hematological profiles respond to parasite infection as a direct result of parasite-induced blood and energy losses (Colditz 2008), up-regulation of host immunity in response to infection, and even the repair of collateral damage caused by host immune mediators (Lochmiller and Deerenberg 2000, Colditz 2008). For instance, some species of gastrointestinal (GI) helminths remove red blood cells from circulation by directly sucking host blood (e.g. barber pole worms (*Haemonchus* sp.) in ruminants) or by inflicting intestinal lesions (e.g. hookworms in humans). This form of chronic blood loss alters the host's hematological profile by reducing red blood cell counts and volume in the short term, and by gradually impairing red blood cell regeneration over the long-term, leading to the production of small, hemoglobin-deficient red blood cells (Harmening 1997). Non-bloodsucking helminths (e.g. *Cooperia* sp. and *Trichostrongylus* sp. in ruminants) can also alter host hematology by limiting essential nutrients (e.g. amino acids,

copper, protein) or as a byproduct of the immune response to chronic infection (Feldman et al. 2000). Ultimately, parasite-induced hematological changes can increase host morbidity and/or mortality (Chambellan et al. 2005, Qiu et al. 2010, Rodrigues et al. 2010), and decrease reproductive output (Bearhop et al. 1999, Allen 2000, Ramakrishnan 2001); Table 1). Thus, examining hematological parameters, in addition to standard body condition indices, may provide an integrated, short-term measure of the effect of parasites on hosts.

In this study, we examined the relationships between multiple GI parasite infections and host hematological profiles and body condition in free-ranging African buffalo (*Syncerus caffer*). Non-invasive GI parasite identification and quantification is typically based on the number and types of infective stages shed in host feces (Ezenwa and Jolles 2008, Turner and Getz 2010), but the infective stages of many GI parasites are similar in appearance and are morphologically identifiable only to order or family-level (e.g. the strongyle nematodes). Recent advances in molecular techniques allow for more detailed identification of parasite eggs and larvae (Zarlenga and Higgins 2001, Gasser et al. 2008, Bott et al. 2009), and here we used a combination of morphology-based aggregate parasite data and molecular-based species-level data to examine the extent to which information on parasite species identity improves inferences about parasite effects on co-infected hosts. Using aggregate parasite data, we examined the direct effects of parasite richness and egg abundance on four distinct hematological parameters indicative of blood oxygen carrying capacity and red blood cell quantity and regenerative ability (see Table 1). We also tested for indirect effects of immune investment, quantified as numbers of lymphocytes, on red blood cell parameters, since immune defenses are energetically costly to develop and maintain, and may indirectly affect host physiology and performance (Moore and Hopkins, 2009). Finally, using species-specific parasite data, we examined the degree to which

parasite identity influenced host costs in terms of hematological profile and immune investment. In all cases, we compared results based on host hematological parameters to results using a more traditional index of host body condition.

Overall, we expected that: 1) aggregate parasite richness and egg abundance would be negatively associated with host physiological indices, reflected by declines in hematological indices and body condition; 2) investment in lymphocytes, an index of innate immune defenses, would be positively correlated with parasite richness and intensity, and show independent negative associations with hematological parameters and condition; and 3) parasite species identity would play a key role in the type and magnitude of observed effects, since the effects of blood-feeding and non-blood feeding parasites are likely manifest through different hematological parameters. Throughout, we expected that if parasites were exerting costs on the host, rather than vice versa, this would be apparent as consistent negative associations between hematology, body condition and parasite infection, or between immune investment and physiological indices. We also expected that parasite species-specific differences in detectable costs would lend support to the idea that observed associations reflect parasite effects on the host rather than host effects on the parasites.

Materials and methods

Background

African buffalo were sampled from Hluhluwe-iMfolozi Park (HIP) and Kruger National Park (KNP), South Africa. In HIP, herds were sampled over two weeks in the Masinda section of the park in October 2005 (dry season) and again in May 2006 (wet season). Buffalo were captured by HIP management by funneling animals into a corral using a helicopter. In KNP, animal capture

was carried out by South Africa National Parks Veterinary Wildlife Services in June/July 2008 (wet season) and October 2008 (dry season). Buffalo herds were located by helicopter and females between the ages of 2-3 were preferentially targeted for darting due to the needs of a concurrent study. Captured buffalo were branded (HIP, KNP) and fitted with radiocollars (KNP) to prevent re-sampling. Overall, 203 males (HIP only) and 278 females (134 from HIP and 144 from KNP) were sampled.

Once animals were captured and immobilized, we collected the same suite of host trait data on all individuals at both study sites including age, sex, body condition and reproductive status (females only). Buffalo age was estimated based on body size and horn development for individuals under 2.5 years old (Sinclair 1977). Incisor eruption patterns were used to estimate age for animals from 2.5 to 5.5 years old. For older individuals (>5.5 years), tooth wear of the first incisor was used to estimate age (Jolles et al. 2005). To assess body condition, we used an integrated body fat score based on the manual palpation and visual inspection of four areas of the body where buffalo store fat (Ezenwa et al. 2009). This body condition index has been shown to be correlated with the kidney fat index, a widely used measure of body condition in ungulates (Ezenwa et al., 2009). Finally, pregnancy status was evaluated via rectal palpation and lactation status was assessed by manual milking of all four teats.

Hematological parameters

We collected blood samples from all individuals for hematological analysis. Samples were collected via jugular venipuncture into 10 ml EDTA tubes and placed on ice immediately after collection until processing. Blood samples from HIP were shipped to Dr. Bouwer & Partners Inc. (Durban, South Africa) and analyzed for hematological parameters and white blood cell

differential counts using an ADVIA 120 automated analyzer. Samples were processed within 3 days of collection. For the KNP samples, we used an ABX ABC Vet hematological analyzer for the hematological measures and samples were processed on the day of collection. Blood smears were made within 8 hours of blood collection and white blood cell differential counts were performed manually by a single observer using a compound microscope. The four red blood cell measures used and their medical and ecological importance are described in Table 2.1. The main effector cells of the adaptive immune system are B and T lymphocytes (Janeway 2008). As such, the proportion of white blood cells that were lymphocytes was used to assess constitutive adaptive immune function.

GI parasite assessment

To assess GI parasite richness and egg abundance, we used fecal samples collected directly from the rectum of each immobilized animal. After collection, samples were placed on ice while in the field and at 4 °C for 0-5 days until processing. GI parasites were quantified following a modified McMaster egg counting protocol (Ezenwa 2003). Five aggregate classes of parasites were distinguished including: *Monezia sp.* (Platyhelminthes: Anoplocephalidae), *Trichuris sp.* (Nematoda: Trichuridae), coccidia (Apicomplexa: Eimeriidae), *Strongyloides sp.* (Nematoda: Strongyloididea), and strongyle nematodes (Nematoda: Trichostrongylidae). We defined parasite richness as the total number of parasite types present in a sample. We used the number of strongyle nematode eggs per gram of feces (egg abundance) as an index of the energetic burden of parasite infection since this was the dominant parasite type, and most of the resources taken from the host by nematodes are converted into eggs (Jennings and Calow 1975, Combes 2001).

For species-specific analyses, we identified strongyle nematodes to species in a subset of the individuals captured in KNP (n = 30). To do this, we isolated third-stage larvae from fecal samples using a modified Baermann technique, which included culturing up to 35 g of feces for approximately 10 days to allow eggs to hatch then collecting individual larvae. Cultures were inspected and stirred every 3 days to prevent fungal growth. After 10 days, culture jars were filled with water and inverted onto a Petri dish. Larvae migrating into the clean water in the Petri dish were collected using a pipette under a stereomicroscope (Archie and Ezenwa 2011). Following isolation, we prepared the larvae for DNA extraction by removing the outer sheath with a 0.15% sodium chlorite solution (Coles et al. 2006). Ex-sheathed larvae were then washed twice and stored in 95% ethanol until further analysis.

DNA was extracted from individual larvae following Archie and Ezenwa (2011). For each larva, we amplified then sequenced the ITS-2 region, an established marker for helminth identification (Heise et al. 1999, Zarlenga and Higgins 2001), and then identified each specimen to species-level using reference samples in GENBANK. A total of 6 to 23 (mean = 10.2) larvae were sequenced from each of 30 buffalo. We identified four nematode species including: *Cooperia oncophora*, *Haemonchus contortus*, *Haemonchus placei*, and *Trichostrongylus axei*. To confirm the classification of *Haemonchus* species, we examined the identity of 3 polymorphic nucleotides in the ITS-2 region known to differ between *H. contortus* and *H. placei* (Stevenson et al. 1995). To calculate the relative frequency of each parasite species per host we divided the number of larvae of each species by the total number of larvae identified. Egg abundance per species was estimated by multiplying the relative frequency of each species by the total egg count per sample (Wilson et al. 2008, Oliveira et al. 2009). *Statistical analysis*

Aggregate parasite data: direct and indirect effects

We used general linear models to examine the direct costs of parasite co-infection on four distinct hematological parameters (see Table 2.1), and the association between parasite co-infection and host body condition. Separate analyses were performed for males and females since different sets of host traits were included in analyses for each sex. For males, the main predictor variables included two measures of infection, GI parasite richness and strongyle egg abundance. Since a number of factors can affect hematological status in buffalo (Beechler et al. 2009), we included the following variables: host age, season captured, and herd affiliation (nested within season) as covariates in our models. For female buffalo, we used the same measures of parasite infection as for males. Likewise, host age and season captured were included as covariates. We also included pregnancy status and lactation status. Since females were captured at two different sites (HIP and KNP), site was included in these models to account for potential differences among locations in host condition and hematological parameters. For both male and female models, GI parasite richness was coded as an ordinal variable since only five parasite types were described. All other variables were continuous. Wilcoxon sign rank tests were used to compare GI parasite richness, strongyle intensity, hematological parameters, and body condition between males and females. For each sex, we ran independent models for body condition and each of four hematological parameters variables (HG, HCT, RBC, MCV). Although the four hematological variables are correlated to differing degrees, they have different biological interpretations and are therefore most informative when considered separately (Table 2.1). Residuals of all models were tested for normality with Shapiro-Wilk tests, and generalized linear models with an inverse Gaussian link function were used in all cases where model residuals were non-normal. When

significant effects of GI parasite richness were detected, differences among levels of richness were examined further using Tukey's multiple comparisons tests.

To examine the indirect effects of infection in terms of immune activation, lymphocytes were first examined as an independent variable in the hematology and condition models described above. Next, lymphocytes were examined as a dependent variable using general linear models with aggregate parasite richness, strongyle egg abundance, and the same covariates as described above for male and female models as explanatory variables. Although eosinophils also play a major role in anti-nematode defenses (Klion and Nutman 2004), they were not associated with any hematological parameter in our preliminary analyses, and therefore were not included in our models.

Species-specific parasite data: direct and indirect effects

We identified four species of strongyle nematodes infecting our subset of 30 females (*Cooperia oncophora*, *Haemonchus placei*, *Haemonchus contortus*, and *Trichostrongylus axei)*. *C. oncophora* infected 96% of individuals; two *Haemonchus* species, *H. placei* and *H. contortus* were the next most common infecting 44% and 24% of sampled buffalo, respectively; and *T. axei* was the most rare, infecting only one individual (4%). For the analyses, we categorized individuals based on the two predominant infection states: *C. oncophora* only infection (*C*-only) and *C. oncophora*-*Haemonchus sp.* co-infection (*C-H*). *Haemonchus placei* and *H. contortus* data were combined for the *C-H* infection group due to sample size (n = 3 *C-H. contortus*, n = 8 *C-H. placei*, and n = 2 *C-Hp-Hc*), and based on previous research suggesting these two species have very similar effects on host physiology (Le Jambre 1995). We used this fine-scale parasite data to examine the effects of both co-infection status and species-specific egg abundance on

host hematological profiles and associations with body condition. For both co-infection status and species-specific abundance analyses, individual host traits were not included as covariates in the models because of the relatively small number of individuals sampled for parasite identification (n = 30 females). However, because exploratory analyses suggested strong effects of pregnancy, but not season or age on response variables, five pregnant individuals were excluded from subsequent analysis.

To examine the effects of co-infection status on the host, we classified animals according to the number of strongyle nematode species harbored, and then used non-parametric Kruskal-Wallis tests to examine differences among uninfected individuals (n = 55 uninfected, non-pregnant females captured at the same study site) and individuals infected with *C*-only (n = 10) or *C-H* (n= 13). Next, we examined the relationships between *Cooperia* egg abundance and *Haemonchus* egg abundance and the same hematological parameters and condition indices. We included only individuals infected with at least one nematode species in the abundance analyses. For individuals infected with *C*-only, *Haemonchus* egg abundance was classified as zero. To examine the detectability of effects without knowing species identity, we also examined the relationships between total strongyle egg abundance and host hematology and body condition. All egg abundance data were log transformed to approach normality, and associations between egg abundance and performance indices were examined using linear regression models. Finally, to examine the indirect costs of infection, associations between total, *Cooperia*, and *Haemonchus* egg abundance and lymphocytes were explored using linear regression models. We also examined the costs of increased immune investment by testing associations between lymphocytes and residual hematological values or body condition score. Residual physiological indexes were calculated from the linear regression of total strongyle abundance versus RBC, HG,

HCT, MCV, or body condition. For both species-specific and aggregate data, all statistical analyses were performed in R (Version 2.10.1, 2009) and SAS (Version 2.1, 2009).

Results

Aggregate parasite data

Sex differences in parasitism, hematological parameters, and condition

Males and females varied significantly in measures of parasitism and hematological parameters; these differences persisted if males were compared to only HIP females. At the aggregate level, parasite richness was higher among males (n = 203 males, 278 females, Wilcoxon's W = 37354, p <0.00001, Fig. 2.1), and males shed significantly more strongyle eggs than females (W = 34419, p <0.00001). Females had higher HG, HCT, and MCV, but lower RBC than males (HG: W = 24035, p = 0.0004; HCT: W = 23831, p = 0.0003; MCV: W = 19719, p < 0.00001; RBC: W = 33694, p = 0.009). However, body condition did not differ between the sexes (W = 29590, p = 0.62).

Direct costs of infection

There were very few associations between aggregate parasite data and measures of hematological status and condition. All four hematological variables were strongly influenced by age, season, and herd affiliation in males (Table 2.2), and in females, site, season, and pregnancy had the most consistent effects on hematological parameters (Table 2.3). In males, body condition was significantly associated with season and herd (nested within season) (Table 2.2), while in females, season, site, pregnancy and lactation status all significantly influenced condition (Table 2.3). After controlling for covariates, we found that parasite richness was

significantly and negatively associated with MCV in males (Table 2.2, Fig. 2.2), however, there was no correlation between richness and either RBC, HG, HCT or body condition (Table 2.2). In females, parasite richness was not a predictor of variation in condition or any of the four hematological variables (Table 2.3). In both males and females, no correlation was detected between strongyle intensity and body condition or hematological parameters.

Indirect costs of infection

When we examined how parasite infection influenced adaptive immune function in males, we found that strongyle egg abundance, but not GI parasite richness, was significantly and positively associated with lymphocyte count after controlling for key covariates (egg abundance: estimate = 0.00004, $F_{1,202} = 8.07$, p =0.005; parasite richness: $F_{3,202} = 0.19$, p = 0.91; age: estimate = -0.019, $F_{1,202} = 43.7$, p < 0.0001; herd (nested within season): $F_{8,202} = 2.00$, p = 0.049; season (dry): estimate = 0.035, $F_{1,202} = 2.55$, p = 0.11). However, for females, there was no association between either parasite measure and lymphocytes (egg abundance: estimate = 0.00004, $F_{1,277} = 1.20$, p = 0.27; parasite richness: $F_{2,277} = 0.17$, p = 0.92; age: estimate = -0.014, $F_{1,277} = 25.9$, p<0.0001; pregnancy: estimate = -0.030, $F_{1,277} = 2.08$, p = 0.15; lactation: estimate = 0.005, $F_{1,277} = 0.04$, p = 0.84; site: estimate (HIP) = 0.078, $F_{1,277} = 12.0$, p = 0.0006; season: estimate (dry) = -0.055, $F_{1,277} = 8.17$, p = 0.005). When we examined the potential effects of lymphocytes on host hematological status and condition, we found that lymphocytes were significantly and negatively associated with HG and HCT, but not RBC, MCV, or body condition in males (Table 2.2). Once again, there was no association between lymphocytes and any physiological parameter in females (Table 2.3).

Species specific data

Direct costs of infection

Co-infection status had only weak effects on hematological variables. Comparing uninfected to *C*-only and *C-H* infected females, we found that no differences among groups for RBC, HG, HCT, or MCV (RBC: $\chi^2 = 2.76$, p = 0.25; HG: $\chi^2 = 5.68$, p = 0.058; HCT: $\chi^2 = 2.03$, p = 0.36; MCV: $\chi^2 = 3.86$, p = 0.15). However, infection status was significantly associated with body condition, with *C-H* co-infected females being in significantly poorer condition than uninfected and *C*-only infected females ($\chi^2 = 9.24$, p = 0.010, Fig. 2.3).

When we analyzed the effects of total and species-specific egg abundance on hematological profile and body condition we found a series of interesting patterns. Across all individuals (n = 23), total strongyle egg abundance was negatively correlated with HCT (r = -0.50 , p = 0.015; Fig. 2.4), but had no detectable effect on RBC, HG or MCV (RBC: r = -0.06, p = 0.77; HG: r = -0.16, p = 0.46; MCV: r = -0.40, p = 0.06; Fig. 2.4, Column 1). When we subdivided total egg abundance by species, we found that *C. oncophora* egg abundance was negatively associated with HCT and MCV (HCT: r = -0.42, p = 0.04; MCV: r = -0.45, p = 0.03, Column 2). However, *C. oncophora* egg abundance was not significantly correlated with either RBC or HG (RBC: r = 0.03, p = 0.87; HG: r = -0.05, p = 0.82; Fig. 2.4, Column 2). On the other hand, *Haemonchus sp.* egg abundance was significantly and negatively correlated with RBC count and HG (RBC: r = -0.46, p = 0.03; HG: r = -0.41, p = 0.048; Fig. 2.4, Column 3), but there was no significant association between either HCT or MCV and *Haemonchus sp.* egg abundance (HCT: r = -0.26, p = 0.23; MCV: r = 0.33, p = 0.12; Fig. 2.4, Column 3). Overall, we found that each nematode species showed strong negative effects on two separate hematological indices (*Cooperia*: HCT and MCV; *Haemonchus*: HG and RBC), but when considered in aggregate (i.e.

total egg abundance), effects were only detectable for a single index (HCT). Similarly, associations with body condition were not detectable when we tested the strength of the relationships between total strongyle egg abundance and condition ($r = 0.38$, $p = 0.077$; Fig. 2.4). However, when the species were considered separately, body condition was negatively correlated with *Haemonchus sp.* egg abundance ($r = -0.55$, $p = 0.007$), and positively correlated with *C. oncophora* egg abundance ($r = 0.52$, $p = 0.011$; Fig. 2.4).

Indirect costs of infection

In terms of indirect effects, investment in lymphocytes did not differ among uninfected, *C*-only infected, and *C-H* co-infected females ($\chi^2 = 4.04$, $p = 0.13$). However, lymphocyte counts were positively correlated with *C. oncophora* egg abundance ($r = 0.55$, $p = 0.006$) and total strongyle egg abundance ($r = 0.49$, $p = 0.018$), but there was no association between lymphocytes and *Haemonchus* egg abundance ($r = -0.28$, $p = 0.19$; Fig. 2.5). In addition, costs of lymphocyte investment were detected with MCV. Lymphocyte investment was also negatively correlated with body condition. Accounting for total strongyle egg abundance, residual MCV was negatively correlated with lymphocyte investment ($r = -0.45$, $p = 0.031$, Fig. 2.5), while residual body condition was positively correlated with lymphocyte investment ($r = 0.44$, $p = 0.038$, Fig. 2.5). There was no association between residual RBC, HG, or HCT and lymphocyte investment (RBC: $r = 0.38$, $p = 0.07$, HG: $r = 0.21$, $p = 0.34$, HCT: $r = 0.09$, $p = 0.68$).

Discussion

Our results suggest that the costs of parasitism are detectable over the short term using physiological performance indices. Specifically, host hematological profiles appear to be

sensitive to variation in infection levels, with parasite species richness and identity largely driving observed direct effects. In addition, we found that indirect costs of infection, due to investment in immunological defenses, were detectable via changes in host hematological parameters. Importantly, our data show that the detectability of both direct and indirect effects depended on the resolution of the parasite data. With aggregated parasite data, physiological effects may only emerge when effect sizes are considerably large, which may explain the differences we found in male compared to female buffalo. Our results also suggest that aggregated data can mask parasite effects because even closely related parasite species may alter host hematological parameters and immunity in different ways and to differing degrees.

Aggregate parasite data: direct and indirect effects

With aggregated parasite data, the direct costs of co-infection were only detectable for males in our study; we found no association between measures of parasitism and host hematological variables in females. In males, GI parasite richness was significantly and negatively correlated with the mean corpuscular volume (MCV) of red blood cells. This pattern of variation in MCV may be indicative iron deficiency and anemia of chronic infection (Jain, 1993). Variation in RBC turnover has previously been linked to both individual survival and reproduction in wild populations (Bearhop et al., 1999; Harvey, 2000; Noyce and Garshelis, 1994). Interestingly, our models show that it was species richness rather than egg abundance that had a significant effect on MCV; and even without knowing parasite community composition, we detected a stepwise decrease in MCV with the number of parasite types. The diversity of the parasite community within an individual host may therefore be an important, yet underappreciated, indicator of the potential magnitude of parasite-induced costs. Similarly,

stepwise effects of helminth species richness on host hemoglobin levels have often been detected in human populations (Ezeamama et al., 2005; Ezeamama et al., 2008; Midzi et al., 2010). These studies support the idea that parasite community richness itself, separate from the intensity of infection, has physiological costs. The costs associated with parasite richness may be particularly potent because the richness of different parasite groups commonly co-vary (Krasnov et al. 2005, Balestrieri et al. 2006, Holmstad et al. 2008). Furthermore, the tendency for particular hosts to have multiple parasite infections may create positive feedback cycles that magnify the net parasite effects on these hosts.

Our aggregate parasite data also show that males experienced greater indirect costs of infection than females. Specifically, lymphocyte counts were negatively correlated with hemoglobin (HG) and hematocrit (HCT) in males, even after accounting for GI parasite richness and strongyle egg abundance. By contrast, there was no effect of lymphocytes on any hematological parameter in females. Males may therefore pay a cost of investing in lymphocytes since low HG and HCT levels are often associated with lower aerobic performance, reproduction, and survival in mammals (Gledhill et al. 1999, Allen 2000). Evidence from livestock and human medicine suggests that the potential fitness costs of declines in HG and HCT can be substantial. For example, Aumont et al. (1991) found that helminth-infected calves had a mean HCT approximately 3% lower than uninfected calves, and also gained 10-15% less weight per day over a 7-month period. Similarly, lower HG levels due to helminth infection have been linked to stunted growth and reduced aerobic performance in human children (Bustinduy et al. 2011). In general, the complex and dynamic nature of the vertebrate immune system makes the fitness costs of immune system maintenance and use difficult to assess, particularly in wild populations (Lochmiller and Deerenberg 2000, Martin et al. 2008). However, lymphocytes are

energetically costly to develop and maintain (Klasing 2004), play a major role in helminth defense (Janeway, 2008), and have been shown to correlate with helminth diversity across mammal species (Bordes and Morand 2009). As such, our results linking lymphocytes to host hematological profiles suggest that the costs of immune deployment may be quantifiable by examining changes in other hematological components, and may offer a potentially new approach for assessing the costs of immunity.

The male-biased direct and indirect costs of infection we observed at the aggregate level may be a function of male-biased infection. In our study population, males had 50% richer GI parasite communities than females, and this may have led to stronger, and therefore more detectable, costs than in females. Male-biased parasitism is common in mammals, particularly for arthropod and helminth parasites and in host species with a high degree of sexual size dimorphism (Poulin 1996, Schalk and Forbes 1997, Moore and Wilson 2002). Furthermore, sex-biases in infection have been linked to biases in parasite-related fitness costs both within and across host species (Gulland 1992, Moore and Wilson 2002, Craig et al. 2009). For this reason, sex-biased infection may help account for the difference in our ability to detect direct hematological costs and indirect immune-mediated costs of parasitism in male versus female buffalo using aggregate parasite data.

Species-specific parasite data: direct and indirect effects

Although no costs of infection were detected in females using aggregate parasite data, significant direct effects emerged once the abundance of individual parasite species was taken into account. In particular, we found that *Cooperia* egg abundance was negatively correlated with MCV and HCT, while *Haemonchus* egg abundance was negatively correlated with HB and

RBC. Indeed, the known effects of *Cooperia* and *Haemonchus* infection in livestock help provide biological explanations for the patterns we observed in free-ranging buffalo. In livestock infected with *Cooperia* or other non-bloodsucking nematodes, impaired red blood cell regeneration has also been described and is believed to be a consequence of impaired digestion and resulting deficiencies in key nutrients, as well as the costs of immune responses to infection (Baker and Douglas 1957, 1966, Soulsby 1982, Feldman et al. 2000). For *Cooperia*-infected hosts, associations between egg abundance, HCT and MCV may indicate impaired oxygen carrying capacity and red blood cell regeneration (Jain 1993). In buffalo, the associations between *Haemonchus* egg abundance, HG and RBC are likely indicative of chronic blood loss and associated costs, including reduced oxygen binding capacity and reduced aerobic capacity (Bustinduy et al., 2011; Jain, 1993). *Haemonchus* is known to cause anemia, emaciation, and even mortality in livestock (Fourie 1931, Le Jambre 1995, Yacob et al. 2008). Further investigation of the consequnces of *Haemonchus* infection in buffalo may be warranted given the similarity of our hematologic findings to those from domestic livestock.

In contrast to the hematological effects described above, the associations we found between parasite egg abundance and host body condition were less clear cut. *Haemonchus* egg abundance was negatively correlated with body condition, while *Cooperia* abundance was positively correlated with condition. These contracting relationships mirrored our analysis showing that body condition did not differ between uninfected and *C*-only infected individuals, but was significantly lower for *C-H* infected individuals. The apparent positive correlation between *Cooperia* egg abundance and buffalo body condition runs counter to observations in livestock linking this parasite to declines in growth and production (Stromberg et al. 2012). Further work in our study system is needed to determine the factors underlying the positive and

negative correlations we observed between body condition and *Cooperia* and *Haemonchus* egg abundance, respectively.

The indirect costs of infection also varied by parasite species in our study. *Cooperia* abundance was significantly and positively correlated with lymphocytes, while *Haemonchus* was not associated with this measure of immune defense. Since *Cooperia* infection is known to induce strong B-cell responses in livestock (Nieuwland et al. 1995, Parmentier et al. 1995), it seems plausible that in buffalo, *Cooperia* may also induce a strong B-cell response, which likely corresponds to an increase in circulating lymphocytes. By contrast, two recent livestock studies found that *Haemonchus* intensity was negatively correlated with lymphocytes (Lacroux et al. 2006, Rowe et al. 2008). Our analysis of indirect costs also revealed a negative correlation between lymphocytes and residual MCV (i.e. MCV corrected for strongyle egg abundance), indicating that investment in lymphocyte defenses may carry a physiological cost detectable by examining red blood cell volume (MCV). Since *Cooperia* egg abundance was directly and negatively correlated with MCV, these patterns collectively support the idea that in buffalo the costs of non-blood sucking nematodes like *Cooperia* may indeed manifest as by-products of immune investment. However, further research is needed to determine the directionality of the lymphocyte-MCV relationship we observed (i.e. lymphocytes affecting MCV rather than red blood cell regeneration influencing lymphocyte numbers). Interestingly, we also found that body condition corrected for strongyle egg abundance (i.e. residual body condition) was positively correlated with lymphocyte investment, possibly indicating that those individuals able to maintain high body condition for their level of *Cooperia* infection may also be able to sustain high lymphocyte defenses. Nevertheless, the same animals may still suffer the indirect costs of high lymphocyte investment as reductions in MCV.

Given that *Cooperia* and *Haemonchus* parasites had different direct and indirect effects on host hematology, it is perhaps not surprising that many of these species-specific effects were masked when total strongyle egg abundance was considered. In fact, overall egg abundance was significantly correlated with only one hematological index, HCT. This may be because the effects of both parasites on HCT were qualitatively in the same direction (i.e. negative effects), whereas for several other parameters, the neutral effect of one parasite may have swamped out the negative effect of the other (Fig. 2.4). In general, our analyses accounting for parasite species identity demonstrate that the costs of infection may be difficult to detect when co-infecting species are considered in aggregate. Reciprocally, the physiological costs associated with individual parasite species may be difficult to assess in the wild due to the presence of co-infecting parasites. Although in our study we could not fully distinguish the effects of each species from co-infection effects since we did not observe any *Haemonchus*-only infections, we were able to infer species-specific costs based on egg abundance data. These species-specific costs provide insight as to why we failed to detect any direct or indirect effects of parasites on females in our aggregate analyses. Thus, it is important to consider the effects of both parasite species identity and parasite community composition when assessing parasite-mediated fitness costs.

Synthesis

Although it is fairly well-established that different GI helminths have distinct effects on livestock hosts (Stear et al. 1998, Bowman 2009), there is still very little known about how individual species within the GI parasite community combine to contribute to the costs of infection in wildlife species. This difference between livestock and wildlife studies arises in large

part because wildlife sampling is often non-invasive with researchers using fecal samples to characterize and quantify GI helminth infection (Wimmer et al. 2004, Turner and Getz 2010, McLean et al. 2012). Determining helminth community composition and relative species abundance from fecal samples is a substantial challenge. Morphological based methods for identifying immature stages of helminth parasites (e.g. eggs and larvae) are not always feasible or are extremely time consuming since differences among species are often minute (Keith 1953, Van Wyk et al. 2004). More recently, molecular techniques have been used to identify both helminth eggs and larvae (Bott et al., 2009; Wimmer et al., 2004; Zarlenga et al., 1998; Zarlenga and Higgins, 2001). In this study we used third stage larvae (L3) for genetic identification because they contain more DNA than eggs and are easier to separate from fecal inhibitors (Gasser et al. 2008). However, the use of larvae instead of eggs may have biased our species composition results due to differential egg viability and larval survival during culture (Dobson 1992) but see (Bryan and Kerr 1989). Nevertheless, our use of a combination of genetic-based identification and traditional egg counts revealed strong patterns that were not detectable with either data set alone. For instance, while there was no relationship between MCV and total strongyle abundance based on traditional egg count data, and no difference in MCV between uninfected and *Cooperia*-infected females based on genetic data, when these datasets were combined, a strong negative relationship between *Cooperia* egg abundance and MCV emerged (Fig. 4). These results highlight a practical way in which traditional egg count data can be combined with molecular data to address key questions about helminth co-infection in wildlife populations where invasive sampling is not feasible.

Our study suggests that parasites impose both direct and indirect costs on their hosts that are detectable using hematological indices. We detected more interactions between GI parasite

infection and hematological parameters than with a traditional measure of energetic reserves, a body condition index based on fat storage. In males, we failed to detect any effects of infection on body condition at the aggregate parasite level, although we detected both direct and indirect effects of infection using hematological indices. Similarly, in our species-specific analysis for females, when parasite species were analyzed in combination, we detected parasite-mediated costs for at least one hematological variable (HCT), but not for body condition. On the other hand, combining species-specific nematode data and egg abundance data revealed strong direct costs of infection on all four hematological variables as well as indirect immune-mediated costs.

The presence of consistent negative associations between hematological indices and egg abundance, and parasite-specific patterns, both support the idea that our results are indicative of parasite effects on the host and not effects of the host on the parasites. Nevertheless, experimental data will be required to establish a definitive, causal link between helminth infection and host hematology. In terms of body condition, species-specific parasite analyses revealed both positive and negative association patterns depending on the parasite, making it difficult to interpret condition effects. These results highlight the difficulty inherent in interpreting parasite-body condition correlations, especially if factors that increase body condition also enhance parasite exposure (e.g. foraging, (Hutchings et al. 2000)), or if the effects of parasites condition on body condition cannot be distinguished from the effects of condition on parasite susceptibility. Taken together, our findings suggest that hematological indices may be a flexible and sensitive tool for assessing the complex relationships among parasites, immune function, and host energy stores than fat-based condition indices. For this reason, hematological profiles may represent a useful tool for assessing the costs of GI parasite infection in wild populations.

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Tables

Table 2.1. Hematological parameters assessed in this study, including the typical response to parasite infection and their reported associations with reproduction and survival in birds and mammals. MCV has a more variable relationship with parasite infection, possibly because it depends on the relative magnitude of changes in RBC and HCT.

Parameter	Definition[12]	Biological Significance[12]	Response to Parasite Infection	Association with Reproduction	Association with Survival
Red blood cells (RBC)	Number of red blood cells in a set volume of blood	Aerobic capacity, blood loss, regeneration	Decrease[1,2]		
Hemoglobin (HG)	Amount of hemoglobin protein in a set volume of blood	Oxygen binding capacity of blood	Decrease[1,2,3]	Positive[4]	Positive[5,6]
Hematocrit (HCT)	Percentage of red blood cells in a set volume of blood	Number and size of RBC, aerobic capacity	Decrease[1,2,3,7,8]	Positive[4]	Positive[9]
Mean corpuscular volume (MCV)	Average size of red blood cells (HCT/RBC)	RBC age and regeneration	Decrease[1] or Increase[2]	Positive[10,11]	

1. (Gill et al., 2007), 2. (Selvam and Baskaran, 1996), 3. (Pfafle et al., 2009), 4. (Allen, 2000), 5. (Chen et al., 2009), 6. (Qiu et al., 2010), 7. (Lotfollahzadeh et al., 2008), 8. (Aumont et al., 1991), 9. (Chambellan et al., 2005), 10. (Bearhop et al., 1999), 11. (Noyce and Garshelis, 1994), 12. (Harmening, 1997)

Table 2.2. Associations between hematological parameters and body condition index and individual host traits for male African buffalo (*Syncerus caffer*) from Hluhluwe-iMfolozi Park (n = 203). Significance level is indicated with asterisks: * < 0.05, ** < 0.01, *** < 0.0001.

	RBC[1]		HG		HCT		MCV[1]		Condition	
Variable	Est.	χ^2	Est.	F	Est.	F	Est.	χ^2	Est.	χ^2
GI Parasite Richness		5.15		1.13		1.01		11.5**		0.59
Strongyle Abundance	0.00001	0.94	-0.00014	0.71	-0.00001	0.81	0.00001	0.02	0.0002	0.67
Lymphocytes	0.0019	2.04	-1.71	4.34*	-0.053	5.82*	0.0001	1.38	-1.03	0.76
Age	0.0002	6.16*	0.13	13.4***	0.0035	13.7***	-0.00001	37.1***	-0.026	0.24
Season	-0.0036	78.7***	1.86	91.1***	0.038	51.7***	0.0001	13.9***	3.24	139***
Herd(season)		39.3***		5.92***		6.47***		30.8***		3.07**

1. χ^2 values from generalized linear models

Table 2.3. Associations between hematological parameters and body condition index and individual host traits for female African buffalo (*Syncerus caffer*) from Kruger National Park (KNP) and Hluhluwe-iMfolozi Park (HIP)(n = 278). Significance level is indicated with asterisks: * < 0.05, ** < 0.01, *** < 0.0001.

	RBC[1]		HG		HCT		MCV		Condition	
Variable	Est.	χ^2	Est.	F	Est.	F	Est.	F	Est.	F
GI Parasite Richness		7.8		2.15		0.29		1.09		1.71
Strongyle Abundance	-0.00001	0.07	0.0004	1.42	0.00004	0	-0.00001	0	-0.0004	0.51
Lymphocytes	-0.0006	0.24	-0.178	0.13	-0.234	0.05	-1.78	2.47	0.068	0
Age	0.0002	21.1***	-0.0007	0.1	0.067	1.8	0.393	55.3***	-0.121	7.4**
Pregnancy	-0.0003	0.68	-0.68	16.5***	1.13	9.28**	1.66	17.9***	1.3	15.5***
Lactation	0.014	9.02**	-0.34	2.97	0.149	0.11	1.77	14.6***	-2.12	29.6***
Season (dry)	0.0018	23.5***	-1	42.1***	0.131	0.15	1.33	13.6***	-5.58	335***
Site (HIP)	-0.0036	65.7***	2.97	266***	-32.5	6444***	-1.27	8.86**	-1.09	9.12**

1. χ^2 values from a generalized linear model

Figure Legends

Figure 2.1. The proportion of male (n = 203) and female (n = 278) African buffalo infected with GI parasite communities of varying species richness.

Figure 2.2. The relationship between gastrointestinal parasite richness and MCV for male buffalo. Least squares means ± 1 standard error are shown. Letters designate significant differences among levels of GI parasite richness.

Figure 2.3. Body condition was lower in *Cooperia-Haemonchus* co-infected buffalo compared to uninfected and *Cooperia*-only singly-infected buffalo. Means ± 1 standard error are shown. Letters denote significant differences among groups.

Figure 2.4. The relationship between total and species-specific strongyle egg abundance and four hematological parameters: RBC (cells/uL x 10^6), HG (g/dL), HCT (%), MCV(10^{-15}L), and body condition for 23 female African buffalo. If total strongyle egg abundance was considered (left column), a significant correlation was detected between egg abundance and HCT and lymphocytes. HCT, MCV, body condition index and lymphocytes were significantly correlated with *Cooperia* egg abundance (center column). HG, RBC, and body condition index were significantly correlated with *Haemonchus* egg abundance (right column). Significance is denoted by a large asterisk, and correlation coefficients (r) are displayed in the corner of each graph with significance level (* < 0.05).

Figure 2.5. Indirect costs of infection demonstrated by the relationships between total and species-specific strongyle egg abundance and lymphocyte investment for 23 female African buffalo (A). After accounting for total strongyle intensity, lymphocyte investment was significantly and negatively correlated with MCV, and positively correlated with body condition (B). Significance is denoted by a large asterisk, the correlation coefficient (r) is noted in the corner of each graph with significance level noted (* < 0.05).

Figures

Figure 2.1

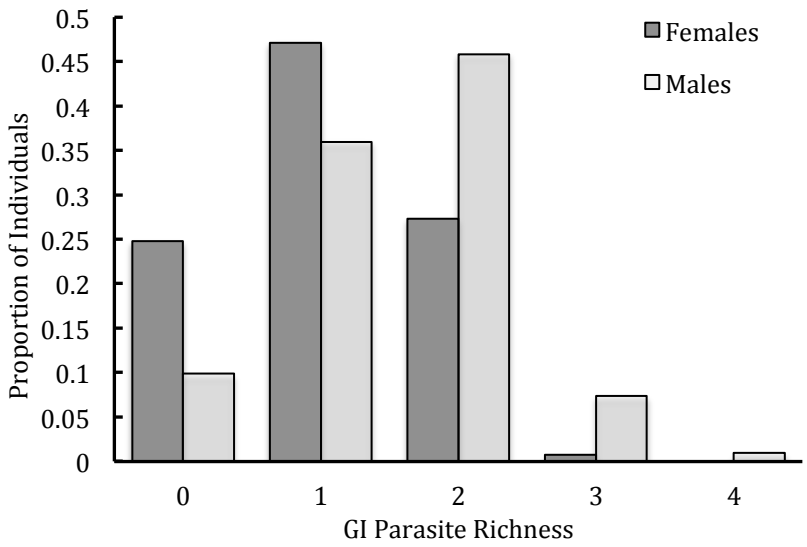


Figure 2.2

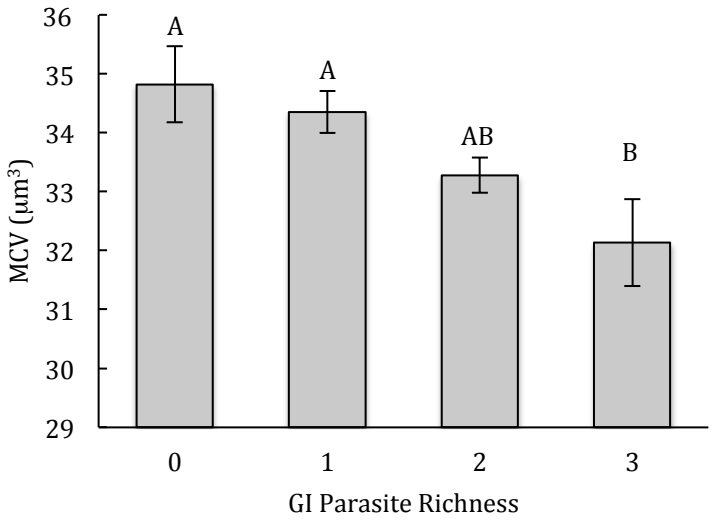


Figure 2.3

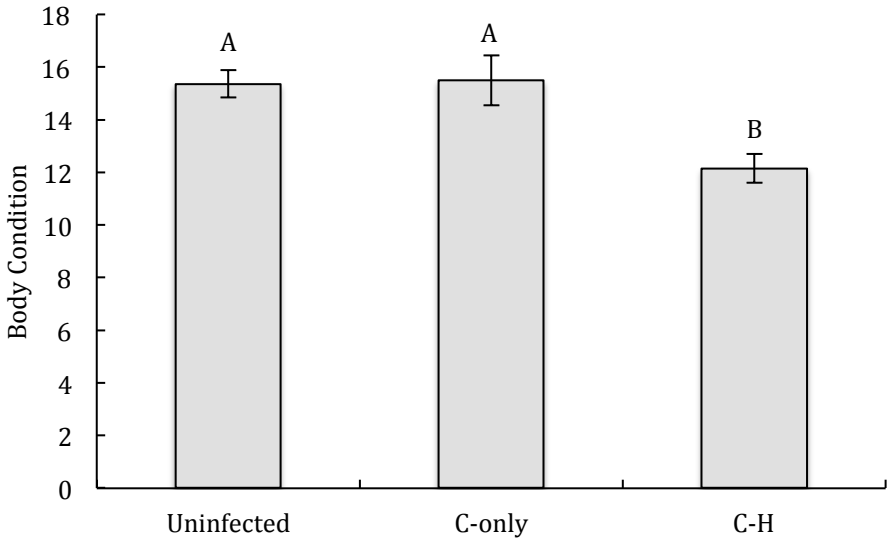


Figure 2.4

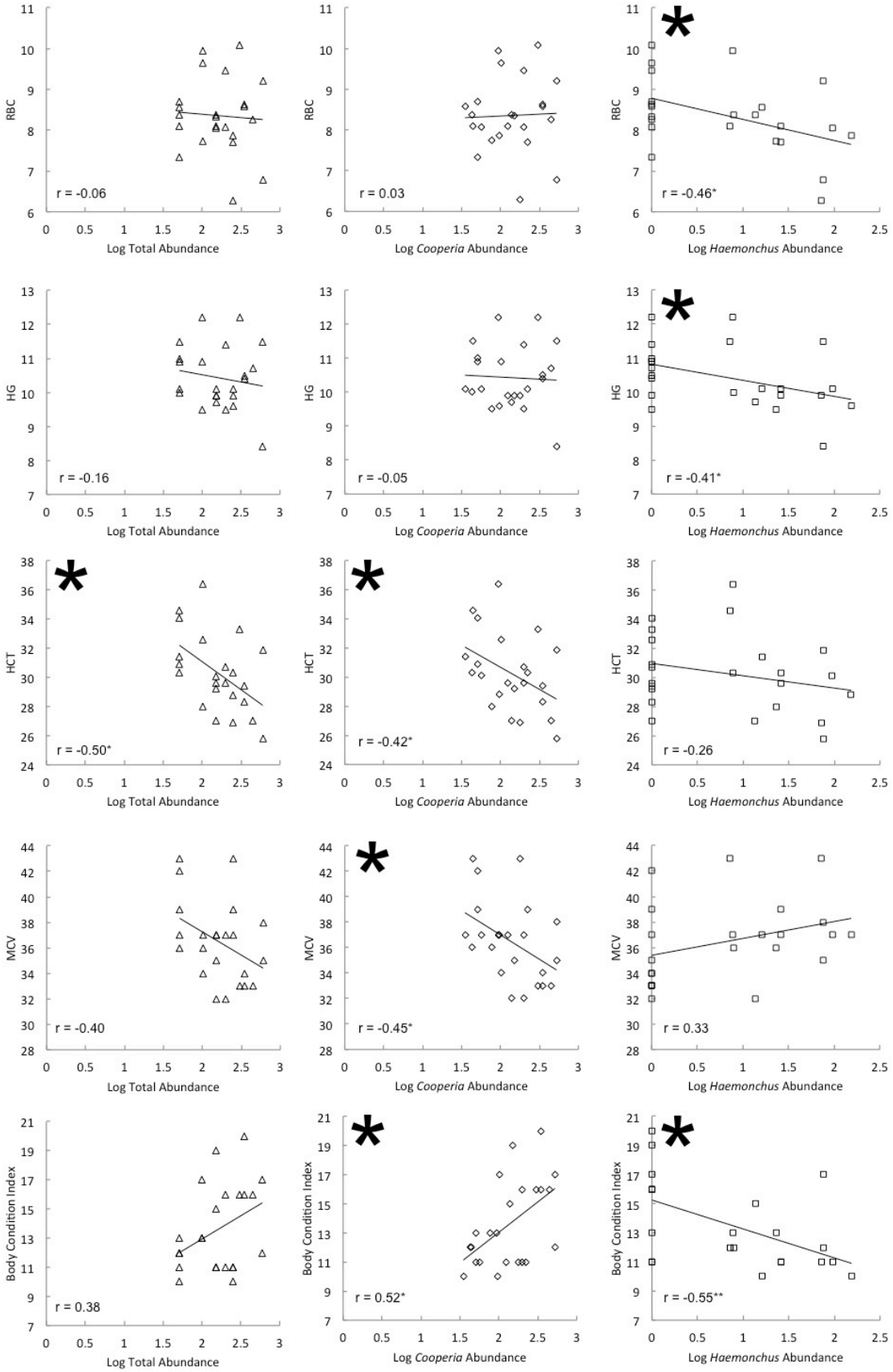
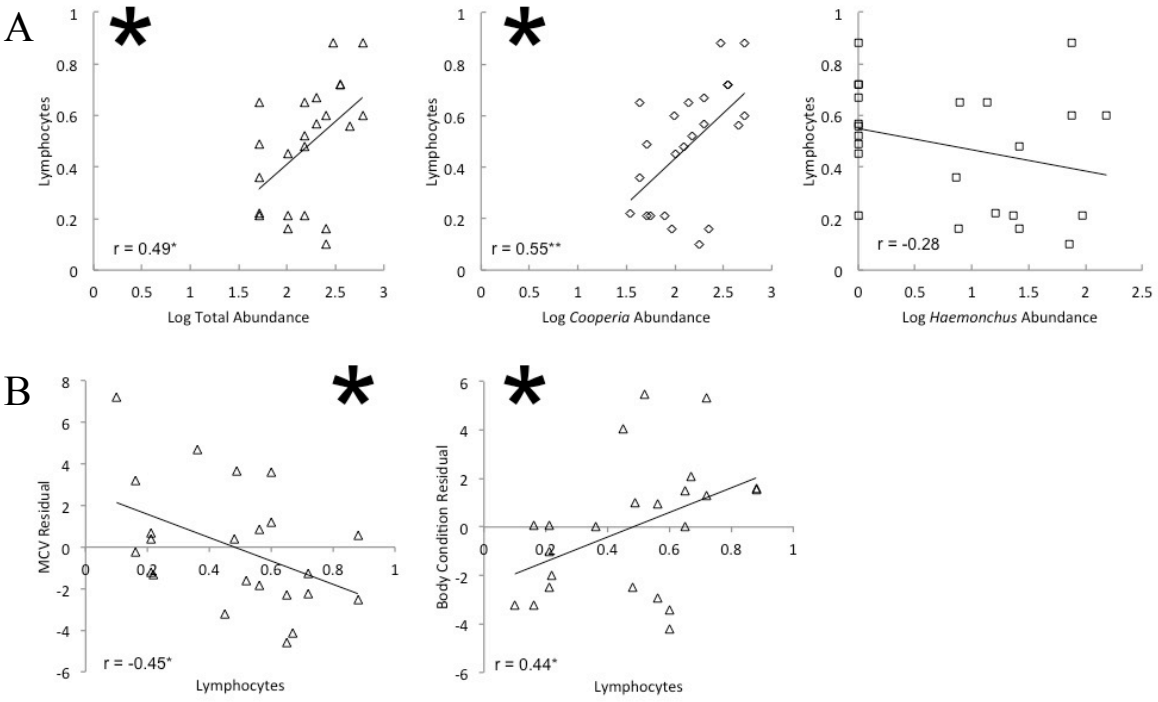

RBC
r = -0.06
Log Total Abundance
RBC
r = 0.03
Log *Cooperia* Abundance
RBC
*
r = -0.46*
Log *Haemonchus* Abundance
HG
r = -0.16
Log Total Abundance
HG
r = -0.05
Log *Cooperia* Abundance
HG
*
r = -0.41*
Log *Haemonchus* Abundance
HCT
*
r = -0.50*
Log Total Abundance
HCT
*
r = -0.42*
Log *Cooperia* Abundance
HCT
r = -0.26
Log *Haemonchus* Abundance
MCV
r = -0.40
Log Total Abundance
MCV
*
r = -0.45*
Log *Cooperia* Abundance
MCV
r = 0.33
Log *Haemonchus* Abundance
Body Condition Index
r = 0.38
Log Total Abundance
Body Condition Index
*
r = 0.52*
Log *Cooperia* Abundance
Body Condition Index
*
r = -0.55**
Log *Haemonchus* Abundance

Figure 2.5


A
Lymphocytes
Log Total Abundance
r = 0.49*
Log *Cooperia* Abundance
r = 0.55**
Log *Haemonchus* Abundance
r = -0.28
B
MCV Residual
Lymphocytes
r = -0.45*
Body Condition Residual
Lymphocytes
r = 0.44*

CHAPTER 3

DISTURBANCE REVEALS THE ROLE OF BIOTIC INTERACTIONS IN PARASITE COMMUNITY ASSEMBLY[1]

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Abstract

Parasite community structure and composition can influence host fitness and parasite transmission, yet there is no consensus regarding factors shaping parasite communities and driving infection heterogeneity in natural populations. To gain insight into the assembly and structure of parasite communities, we experimentally disturbed parasite communities of African buffalo (*Syncerus caffer*) by treating hosts with anthelmintic drugs. We also validated non-lethal sampling methods that can facilitate future studies of parasite community structure in a wide range of host systems. Multiple lines of evidence suggested that parasite communities in untreated, control animals appeared to be randomly assembled, without evidence for species interactions, whereas biotic interactions appeared to play a role in structuring treated communities. Moreover, treated communities increasingly resembled control communities with time since disturbance. These findings suggest an important temporal sequence to the relative contribution of biotic vs. random factors in driving parasite community structure. While non-equilibrium dynamics and random processes have been postulated as explanations for the inconsistencies in parasite community structure within and across species, this is the first study to link recolonization period to the degree of parasite community interactivity.

Introduction

The structure of ecological communities can vary along a continuum from randomly assembled (also called isolationist) to interactive (i.e. assembled via biotic interactions). Isolationist communities have little or no interaction among component species and are characterized by low colonization rates, low species diversity, and a relatively low degree of evenness. Reciprocally, interactive communities have high levels of interspecific interactions and

are characterized by high colonization rates, high species diversity and high evenness (Holmes and Price 1986). Understanding how communities are structured and rules that govern community assembly, can provide insight into the resistance of communities to invasion and resilience following disturbance (Thrush et al. 2009, Martin and Wilsey 2012, Vanschoenwinkel et al. 2013). For example, interactions among recolonizing species will generate similar community compositions across highly structured communities, while random assembly processes will generate heterogeneous compositions across unstructured communities.

Most hosts harbor more than one parasite species, and the structure of parasite communities within hosts can reveal information about the host response to infection, host susceptibility to invasion by new parasites, and the transmission of component parasites (Behnke et al. 2005, Craig et al. 2008, Telfer et al. 2010). Whereas most free-living animal communities are highly interactive, past work showed that the structure of parasite communities varies widely (Cornell and Lawton 1992, Gotelli and Rohde 2002, Krasnov et al. 2011), with some communities appearing to be randomly assembled and others showing evidence of interspecific interactions. These interspecific interactions arise from competition for space and nutrients, as well as via immune-mediated mechanisms. Indeed, the degree of overlap in resource use and strength and type of immune responses that the parasites elicit have been associated with the degree of interactivity in laboratory co-infection studies (Graham 2008). While experiments often detect strong interspecific interactions between coinfecting parasites, there is no consensus regarding community interactivity and assembly processes that shape parasite community structure in wild populations. In particular, the relative importance of random factors, host physiology, host behavior, and interspecific interactions among coinfecting parasites, remain poorly understood despite decades of research focused on identifying factors accounting for

variability in parasite community structure. (Poulin 2001, Kennedy 2009, Viney and Graham 2013).

Studying parasite community structure and assembly is difficult because lethal sampling is often necessary to collect parasite community data, and thus precludes observation of parasite community dynamics. Furthermore, there is no consensus for how to best infer assembly processes from species co-occurrence patterns from such single time-point studies, although analytical methods are improving (e.g. (Timi and Poulin 2007, Fenton et al. 2010). In free-living species, perturbation studies are the 'gold standard' for exploring the factors that drive community assembly (Ernest et al. 2008). These studies can be used to distinguish between the contributions of random, biotic, and abiotic factors to structuring communities (e.g. (Martin and Wilsey 2012). For instance, the strength of interactions between pairs of species or the competitive and colonization abilities of individual species within a community can be quantified from changes in species richness and abundance following a disturbance (e.g. (Jiang and Patel 2008). For instance, the colonization ability of plant species is frequently determined by comparing the relative abundance of species within recently disturbed patches to their frequency in the surrounding vegetation (Bullock et al. 1995, Cichini et al. 2011). Several studies have applied anti-microbial treatments to uncover processes that shape the assembly and structure of microbial communities within multiple host species (Engel and Moran 2013, Krediet et al. 2013, Lozupone et al. 2013). As an example, McFrederick et al. (2014) used a fungicide treatment of bees and comparisons of fungal species richness in treated and control individuals to show that one pathogenic fungal species was competitively dominant in bee gut communities. Since the dominant fungus out-competes other species, the disturbance experiment revealed that biotic interactions play a strong role in bee fungal community assembly, and that a rich fungal

community will not protect bees from invasion by pathogenic species. This interactive community structure and its implications for susceptibility to invasion would be miscategorized by a cross-sectional study since the lower species richness of equilibrium fungal communities suggests a randomly-assembled, isolationist community structure.

For metazoan parasites, anthelmintic treatment studies are increasingly used to study host-parasite interactions in wild populations (Hudson et al. 1998, Craig et al. 2008, Pedersen and Greives 2008, Ezenwa et al. 2010). These studies can provide novel insights about the assembly and structure of parasite communities, but relative few record treatment effects on the abundance and prevalence of non-target parasite species (Knowles et al. 2013, Pedersen and Antonovics 2013), and almost none evaluate the consequences for entire parasite communities (e.g. (Craig et al. 2009). In wild host populations, the structure of helminth communities in terms of the distribution of parasites among hosts (e.g. β diversity) and within hosts (e.g. richness) can vary substantially in space and time (Poulin and Valtonen 2002, Timi and Poulin 2003, Behnke et al. 2005). This observed spatial and temporal variation in parasite community structure could result from non-equilibrium dynamics or from changes in the mechanisms regulating parasite populations (Kennedy 2009). Importantly, these differences in community structure can have immediate effects on host susceptibility, performance, and fitness (Behnke et al. 2005, Craig et al. 2008, Telfer et al. 2010).

Here, we used an anthelmintic treatment-induced disturbance to examine the assembly processes that shape parasite community structure in wild African buffalo (*Syncerus caffer*). Specifically, we tested the effects of disturbance on within-host parasite community structure at two scales: 1) across the entire population of treated vs. control hosts (component community level) and, 2) within individual hosts (infracommunity level). At each scale, we evaluated a suite

of characteristics to determine whether helminth communities from treated and control hosts were isolationist or interactive (Table 3.1). We tested four alternative predictions:

1) Parasite communities from both control and treated hosts will appear isolationist because biotic interactions among co-occurring parasites are weak.
2) Parasite communities from both control and treated communities will appear interactive because biotic interactions among co-occurring parasites are strong.
3) Parasite communities from control hosts will appear isolationist because biotic interactions are weak when communities are at equilibrium, while communities from treated hosts will appear interactive because biotic interactions are strong during the non-equilibrium recolonization phase.
4) Parasite communities from control hosts will appear interactive because the high abundance of parasites within hosts generates strong biotic interactions, while communities from treated hosts will appear isolationist because the low abundance of parasites within hosts generates weak biotic interactions.

For predictions 3 and 4, we also expected the structure of communities from treated hosts to increasingly resemble that of control communities with time since disturbance. Finally, since the need for lethal sampling has significantly hampered progress in our understanding of helminth community structure in wild populations, we tested whether similar inferences about parasite communities could be drawn using non-lethal sampling techniques (i.e., examining parasite transmission stages in feces) relative to lethal techniques (i.e., examining adult parasites *in situ*).

Materials and methods

Sample collection

As part of a longitudinal study of parasite interactions, 269 female African buffalo (*Syncerus caffer*) were sampled from June 2008 to August 2012 in Kruger National Park, South Africa. The first 200 animals were initially captured in groups between June and October, 2008. Additional animals were added to the study to replace individuals removed due to death or emigration. Replacements were added between April 2009 and March 2011. Initial group captures were conducted by South African National Parks (SANParks). Animals were immobilized with M99 (etorphine hydrochloride) and ketamine darts from a helicopter, and assigned randomly to the treatment or control group. Treated animals were given a slow-release fenbendazole bolus (Panacur, Hoechst-Roussel, Netherlands) delivered to the rumen known to be highly effective against Trichostrogyle nematodes in livestock. After data collection, immobilization was reversed using M5050 (diprenorphine). Replacement animals were captured by vehicle using the same immobilization and reversal procedure, and were assigned the same treatment status as the individual being replaced. After initial capture, recaptures of all animals occurred at approximately six-month intervals. Recaptures were conducted by vehicle as described above and treated animals were re-dosed with fenbendazole. In June-August 2012, a subset of study animals (n = 134) were euthanized following the SANParks Standard Operating Procedure for Lethal Population Control.

For parasite sampling, a fecal sample was collected from the rectum and the abomasum (Abo) and small intestine (SI) were tied off in the field, removed, and transported back to the laboratory. Fecal samples were used to determine strongyle (Nematoda: Trichostrongylidae) fecal egg counts, performed following a modified McMaster technique (Ezenwa 2003). Thirty-

three individuals were selected for adult helminth sampling based on positive strongyle fecal egg counts. Nine of the individuals had been treated with the broad-spectrum anthelmintic at the previous capture, 3-11 months prior. When administered at sufficient dosage, fenbendazole is 100% effective at killing adult and immature stages of most trichostrongyle nematodes including the two most common genera found in African buffalo, *Cooperia* and *Haemonchus* (Williams 1991, Miller and Morrison 1992, Williams et al. 1992, Williams and Broussard 1995, Garg et al. 2004), although 98% effectiveness against *Cooperia* was documented in one study (Williams 1991). All cattle dosed with the same slow release fenbendazole bolus used in this study remained trichostrongyle egg-free for at least 10 weeks post-treatment (Bauer et al. 1997). Additionally, these trichostrongyle nematodes cannot reproduce within the host. Thus, all observed infections likely represent unique reinfection events. To collect adult nematodes from the 33 infected hosts, the Abo and SI were rinsed using 6 L or 12 L of water, respectively, following standard procedures (Wood et al. 1995). Contents were mixed, and a 2.5% aliquot was removed and preserved in 5% phosphate buffered formalin (PBF). Formalin-fixed adult helminth specimens were isolated from GI contents by rinsing samples through 250 μm and 44 μm sieves. Specimens were counted and morphologically identified at the USDA Agricultural Research Service, Animal Parasitic Diseases Laboratory. Counts for each 2.5% sample were multiplied by 40 to estimate total abundance. Seven species of strongyle nematodes were detected; for one genus (*Haemonchus*), females of two species could not be differentiated morphologically.

Genetic analysis of adult specimens was used to obtain sequence information for species-specific ITS-2 markers used for the identification of larvae collected non-lethally (Heise et al. 1999). Specimens used for these analyses were obtained by pooling aliquots of Abo and SI contents and then isolating worms following an agar-gel procedure (Slotved et al. 1996). Briefly,

pooled samples were mixed with an equal volume of 2% agarose, and then poured in a thin layer over a cloth sheet. Sheets were hung in warm phosphate buffered saline (PBS) overnight to facilitate helminth migration out of the agarose. Specimens were collected by allowing them to settle, pouring off the PBS, and then preserving them in 70% ethanol. Morphological identification occurred at the USDA, followed by genetic analysis at the University of Georgia. For genetic analysis, DNA was extracted from the midbody region of morphologically-identified adult specimens following (Archie and Ezenwa 2011). ITS-2 was amplified using nested PCR reactions (Sim et al. 2010) and sequenced.

Sampling of adult helminths requires lethal sampling, but helminth larvae can be collected and identified using sub-lethal, molecular techniques. To validate this new molecular technique in a wild population, we examined the correspondence in community structure between lethal and non-lethal sampling methods. To sub-lethally sample helminths, we collected fecal samples directly from the rectums of euthanized animals (see above). We then cultured between 1-50 g of each fecal sample for approximately 10 days to isolate third-stage larvae. Cultures were performed using a modified Baermann technique, and isolated larvae were ex-sheathed with a 0.15% sodium hypochlorite solution then stored in 95% ethanol until further analysis (Archie and Ezenwa 2011). To identify larval specimens to the species-level, we extracted DNA from individual larvae (Archie & Ezenwa 2011), then amplified and sequenced the ITS-2 region (Sim et al. 2010). Species-level identifications were done by comparing larval sequences to those from morphologically-identified adult specimens. To increase the sample size for comparison of species richness rarefaction curves for lethal and sub-lethal techniques, the composition of the helminth communities of an additional 119 buffalo that were captured over

the course of the longitudinal study were also identified using larval culture, PCR, and sequencing.

Data Analysis

Among-host community structure analysis

Although interactions among parasites occur at the within-host (infracommunity) level, the structuring of the community at the among-host (component community) level can provide insight into the structure and assembly of within-host communities (Table 3.1). First, to evaluate the effects of disturbance on individual helminth species, we compared species-specific proportional abundance and prevalence between the treated and control groups. Abundance is defined as the number of individuals of a given parasite species within a host, regardless of whether the host is infected, whereas prevalence refers to the proportion of infected hosts (Bush et al. 1997). Next, to assess effects on among-host community structure, we calculated total richness (γ diversity) for the treated and control host groups by summing all helminth species found across individuals of each group. To gain insight into the processes influencing total richness, we quantified the relative contributions of among-host and within-host richness to the total. Average within-host richness (α diversity) was calculated for each treatment by taking the mean of the number of species observed in each host. Among-host richness (β diversity) was calculated for each host by subtraction ($\gamma = \alpha - \beta$), and averaged for each treatment.

For each host group, we also examined the rate of helminth species discovery and how observed richness varied with the number of hosts sampled and the number of parasites sampled. Rarefaction curves were calculated using Jackknife1 estimates (Colwell 2013), a robust, non-parametric estimation technique (Dove and Cribb 2006). To characterize within-host community

interactivity based on among-host structure, we determined the point where 50% of total richness was observed (CC_{50}) by plotting cumulative percent richness by the percent of hosts. The CC_{50} is highly correlated with the degree of interactivity of parasite communities (Poulin and Luque 2003).

Within-host community structure analysis

At the within-host scale, we tested for effects of disturbance on the relative abundance of individual helminth species, and then examined impacts on four measures of community structure: total abundance, richness, diversity, and evenness. We also explored recolonization patterns, by evaluating the relationship between time since disturbance and community structure in treated hosts. For richness analyses, the two *Haemonchus* species were considered separately, but for analyses that rely upon abundance data, these species were pooled to accommodate the female specimens in the totals. We calculated Simpson's (inverse) diversity indices and evenness (Simpson 1949, Magurran 2004) for the community within each host.

Shapiro Wilk tests on model residuals showed that species-specific and total abundance, diversity, and evenness were not normally distributed so Wilcoxon sign rank tests were used to compare treatment and control groups. Richness was normally distributed so general linear models (GLM) were used to test for differences between groups. To examine recolonization dynamics of treated hosts, we calculated the interval between the previous capture when individuals were dosed with the drug and the date they were culled to sample adult helminths. We tested for an effect of capture interval on indices of parasite community structure using linear regression since model residuals were normal. We excluded a single buffalo recaptured within a

month of treatment since that interval was insufficient for full effectiveness of the drug, and this point was an extreme outlier in terms of adult worm diversity and evenness.

Lethal vs. non-lethal sampling comparison

We used matched lethal (adult-based) and non-lethal (larval-based) community data to examine the effects of sampling technique on community structure estimates. Eight host individuals were used for these analyses. Due to life history differences, the larval collection method precluded collection of *Parabronema* sp. larvae, so *Parabronema* sp. was excluded from comparisons. Also, two *Haemonchus* species were combined for these analyses since adult female specimens could not be differentiated.

At the among-host level, we asked whether the mean difference in prevalence of each parasite species differed from zero using a paired sample t-test. Next, we determined the relationship between the number of hosts sampled and richness by calculating a Jack-knife 1 species accumulation curve (Colwell 2013). We compared this larvae-based species accumulation curve to those based on lethal sampling.

At the within-host level, we compared the following indices by sampling method: 1) prevalence, 2) total abundance by host group (treatment vs. control), 3) effects of sample size on proportional abundance and richness estimates, 4) species-specific abundance, and 5) community similarity. We calculated the difference in prevalence of each worm species between adult and larval data, then used a t-test to determine if it differed from zero. To compare total abundance, we examined the correlation between adult worm intensity and fecal egg counts (FEC) for all buffalo combined, and for treated and control animals separately (with the exception of two hosts with insufficient fecal samples for measuring FEC). Pearson correlation tests were used to

examine the adult abundance-FEC relationship separately for treated and control buffalo, but the data were not normal when combined so a spearman correlation test was used to determine the overall relationship between intensity and FEC.

To test if the number of larvae identified (subsample size) influenced the accuracy of proportional abundance estimates, we compared the number of larvae identified to the absolute value of the difference in proportional abundance (adult % - larvae %). Since only two species (Cf and H) were detected using both sampling methods and their proportional abundances sum to 1, the absolute value of percent difference reflects dissimilarities for both species. To further assess the effects of sample size on estimates of species richness and proportional abundance, we pooled the 8 matched samples with additional non-lethally sampled buffalo (with no corresponding adult data) for a total sample size of 127 hosts. Next, we used bootstrapping (n = 1000 replicates) to calculate within-host richness and 95% confidence intervals for each host at a range of subsample sizes (5 to 45 larvae), and then examined how mean within-host richness and the mean difference between observed and bootstrapped richness varied with subsample size (5 to 45 larvae). To examine the effects of subsample size on species-specific proportional abundance (% of larvae identified as each species), we compared the mean differences between observed and bootstrapped values across a range of subsample sizes for each common species and the rare species (Other). We identified a cutoff sample size (n =10 larvae) below which relative abundance estimates were inaccurate.

To compare species-specific abundance, we used hosts with at least 10 larvae identified to test if lethal, adult worm counts of Cf and H were correlated with non-lethal, larvae-based abundance estimates using GLM. The larvae-based abundance estimates were calculated by multiplying the proportional abundance of each species by the total fecal egg count per sample

(Oliveira et al. 2009, Budischak et al. 2012). Lastly, to compare community similarity between lethal and non-lethal methods, we calculated two separate matrices of pair-wise Bray-Curtis dissimilarity indices based on the abundance of shared species for the 8 communities measured using both methods. We calculated the pair-wise dissimilarity matrices with both separate and combined *Haemonchus* species abundances, and then compared lethal and non-lethal community dissimilarity matrices using Mantel tests (Oksanen et al. 2013).

Results

Among-host community structure

Buffalo were found to be infected with 7 species of strongyle nematodes including: *Haemonchus placei* (Hp), *Haemonchus bedfordi* (Hb), *Cooperia fuelleborni* (Cf), *Parabronema sp.* (P), *Trichostrongylus sp.* (T), *Africanastrongylus giganticus* (Ag), and *Africanastrongylus buceros* (Ab). Relative abundance patterns of adult worms were similar across both treated and control hosts, with Cf being the most abundant species identified in both groups (Fig. 3.1). H and P were the next most abundant species, while T and both *Africanastrongylus* species (Ag, Ab) were rare (Fig. 1). Prevalence (i.e. the percent of hosts infected) varied by worm species ($F_{6,6}$ = 45, $p < 0.001$), but not by host treatment status (ANOVA: $F_{1,6} = 0.13$, $p = 0.73$). For both treated and control hosts, the most numerous species, Cf and P, were also the most prevalent. Both Cf and P infected 88% of treated hosts and 96% and 75% of untreated hosts, respectively. Hp and Hb males infected 38% and 50% of treated buffalo; since females of the two *Haemonchus* species could not be differentiated morphologically, the two species were pooled to calculate overall H prevalence at 88% for both treated and untreated buffalo. The remaining parasites were rare in both treated and control hosts, with T infecting 8.3% of control hosts and no treated

animals, Ab infecting 4.2% of control and 13% of treated animals, and Ag infecting 4.2% of treated hosts and no control animals. Overall, among-host level patterns of parasite prevalence were not significantly affected by anthelminthic treatment.

Parasite richness did not differ significantly between control and anthelminthic-treated (disturbed) communities of adult worms. Control communities had a total richness (γ diversity) of 7 species, while treated communities were comprised of 5 species. The two species missing from disturbed communities, T and Ag, were also among the most rare species in terms of prevalence in control hosts. Within-host richness (α diversity) contributed more to total community diversity than among-host richness (β diversity) for treated communities ($\alpha = 3$, $\beta = 2$). Conversely, control communities varied more among hosts than within hosts ($\alpha = 2.88$, $\beta = 4.12$).

Rarefaction curves showed that the rate of adult worm species discovery differed by host treatment status, and by whether the worm specimens were clustered by host or considered individually. Jackknife estimates of host-based species richness were similar for treated and control hosts with both showing an accumulation of approximately 6 worm species with a sample size of 9 hosts (Fig. 3.2a). Since our sample size of control hosts was larger than for treated hosts, the estimated community richness for the control group continued to rise as the number of sampled hosts increased, reaching an estimated maximum richness of approximately 9 species with 24 hosts sampled (Fig. 3.2a). Accumulation estimates based on the number of individual worms sampled showed that richness increased more quickly for the treated group than the control group (Fig. 3.2b). For example, it took sampling 579 individual worms from treated animals to find 5.7 species, but nearly three times that number (1505 individuals worms) to reach the same estimated number of worm species in controls (Fig. 3.2b).

To compare the rate of species accumulation between control and treated hosts independent of sample size and total richness, we calculated the percent of hosts needed to accumulate 50% of the among-host community richness (CC_{50}). Similar to the individual-based rarefaction curve, treated hosts accumulated species faster than control hosts (Treated: CC_{50} = 0.167; Control: CC_{50} = 0.563; Fig. 3.2c). High CC_{50} values are indicative of isolated communities where parasites are distributed randomly across hosts, while low values are indicative of interactive communities where parasites are non-randomly distributed among hosts (Poulin and Luque 2003).

Within-host community structure

The antihelmintic treatment reduced total helminth abundance, such that control buffalo were infected with almost 4-fold more adult worms per sample (Shapiro Wilk test: Treated: 73± 31, Control: 304 ± 66, W = 31.5, $p < 0.001$). When extrapolated to the entire GI tract, this difference equates to an average of over 9,000 more adult worms per untreated animal (Fig. 3.3a). This difference was largely due to the abundance of Cf worms; communities within control hosts had over 9,000 more Cf worms than treated hosts (Shapiro Wilk test: W = 33, p = 0.007; Fig. 3.4). No differences in the abundance of the other strongyle species were observed (H: W = 98, p = 0.95; P: W = 121, p = 0.289; T: W = 88, p = 0.44; Ab: W = 104, p = 0.44; Ag: W = 92, p = 0.61; Fig. 3.4). In terms of community structure, within-host species richness (α diversity) ranged from 2-5 species for treated hosts and from 1-4 species for control hosts, but mean richness did not differ significantly between groups (GLM: t_{30} = -0.35, p = 0.75, Fig. 3.3b). However, on average, treated hosts had higher Simpson diversity indices than control hosts (W = 119, p = 0.032; Fig. 3.3c). Similarly, evenness was lower in treated than control hosts (W = 125,

p = 0.013; Fig. 3.3d). Thus, although richness was similar, treated communities were less intense, had higher Simpson diversity, and were more even than control communities.

Treated communities more closely resembled control communities with increasing time following disturbance. Adult helminth intensity increased with time since last capture (GLM: R^2 = 0.45, t = 2.61, p = 0.04), reflecting an estimated 35.3 ± 13.5 new infections per treated buffalo per day (Fig. 3.5a). There was no relationship between capture interval and richness (R^2 = 0.09, t = -0.78, p = 0.47; Fig. 3.5b), but capture interval was negatively correlated with Simpson diversity (R^2 = 0.80, t = -4.43, p = 0.007; Fig. 3.5c). Capture interval was also negatively correlated with evenness, although this difference was not statistically significant (R^2 = 0.38, t = -2.18, p = 0.08; Fig. 3.5d), suggesting that communities may become less even over time. These patterns were driven by higher Cf intensity in hosts with a greater time lag since treatment, suggesting that hosts disproportionately accumulate Cf over time (Fig. 3.5e). The intensity of H and P did not vary with capture interval (Fig. 3.5e). Overall, these analyses show that adult worm communities in treated hosts begin to closely resemble control host communities as the time since disturbance increases. By approximately 300 days post treatment, richness and evenness reached the average levels for control hosts, although intensity remained lower than the mean for control communities (Fig. 3.5).

Non-lethal sampling – Among-host community structure

The proportional abundance of helminth species was similar when we compared the adult-based, lethal sampling method to the larval-based, non-lethal method for the eight host communities sampled using both methods (Supl. Fig. 3.1). All but one worm species (T) detected by lethal sampling of adults, was detected by non-lethal sampling of larvae. The most prevalent

species detected using non-lethal sampling techniques was Cf, present in 100% of both treated and control buffalo, similar to prevalence observed from destructive sampling. Non-lethal samples tended to underestimate the prevalence of the H (non-lethal: 38% , lethal: 100%). When examined separately, the prevalence of Hp and Hb were also lower in non-lethal samples (Hp: 63%, Hb: 25%), compared to lethal samples (Hp: 38%, Hb: 13%). However, these differences in prevalence were not significant (paired-t test: $t_3 = -2.78$, $p = 0.07$). With regard to community structure, across a larger sample of 127 non-lethally sampled hosts, among-host richness increased with the number of hosts sampled at a rate similar, but slightly slower than for lethal sampling methods (Fig. 3.2d).

Non-lethal sampling – Within-host community structure

A threshold of 10 larvae per host was determined as a cutoff for accurately estimating proportional abundance of each species within a host, based on comparison between lethal and non-lethal methods (Fig. 3.6a). As the number of larvae identified per host increased, within-host richness increased (Fig 3.6b), and the number of species missed (observed - bootstrap estimate) decreased (Fig 3.6c). At the cutoff sample size of 10 larvae, within-host richness is likely underestimated by only 0.61 species (95% CI: 0.54 - 0.68 species). The error in estimating proportional abundance of each species was small across all sample sizes, and variability decreased as the number of larvae identified per host increased (Fig 3.6d). Overall, non-lethal proportional abundance, richness, and species-specific proportional abundance estimates were reliable above a minimum sample size of 10 larvae per host.

Estimates of total abundance from lethal (adult worm counts) and non-lethal (fecal egg counts) techniques were highly correlated overall (Spearman regression: $r_s = 0.76$, $S = 1066$, $p <$

0.0001); and for control buffalo (Pearson regression: $r = 0.84$, $t_{20} = 6.87$, $p < 0.0001$; Fig. 3.7a), and treated buffalo (Pearson regression: $r = 0.74$, $t_6 = 2.70$, $p = 0.035$; Fig. 3.7a). Dropping a single control individual with a high level of infection (60,000 worms) from the analysis did not change the overall or control FEC-adult intensity relationships (overall: $r_s = 0.74$, $S = 1068$, $p < 0.0001$; control: $r = 0.73$, $t_{19} = 4.61$, $p = 0.0002$).

Adult worm counts were significantly correlated with larvae-based abundance estimates for Cf ($r = 0.75$, $t_6 = 2.74$, $p = 0.034$; Fig. 3.7b), and the relationship between these estimates were marginally significant for H ($r = 0.69$, $t_6 = 2.36$, $p = 0.056$; Fig. 3.7c). Importantly, the non-lethal method detected comparable patterns of community dissimilarity as did the lethal method. Pairwise Bray-Curtis dissimilarity indices based on adult and larvae sampling were significantly positively correlated whether the two *Haemonchus* species were combined (Mantel test: $r = 0.32$, $p = 0.014$) or considered separately ($r = 0.32$, $p = 0.018$).

Discussion

Disturbance by anthelmintic treatment influenced gastrointestinal helminth community composition and structure at both the among-host and within-host scales, allowing us to test key predictions about the forces shaping parasite communities. All but one community metric (within-host abundance) suggested that control communities are isolationist, while disturbed communities are interactive. Moreover, treated communities increasingly resembled control communities with time since disturbance, revealing an important temporal sequence where biotic interactions are important in community assembly following disturbance, but random factors play an increasingly important role over the course of succession. Temporal changes in community structure over time were largely driven by an increase in the intensity of a single

dominant parasite species (*Cooperia fulleborni*), suggesting that treated communities preferentially accumulated Cf, rather than randomly accumulating species. Temporal variability in the mechanisms regulating parasite communities has been postulated as an explanation for why parasite community structure is notoriously variable over time and space (Kennedy 2009), and this is the first study to provide evidence for this idea.

At the among-host community scale, species-specific parasite richness was similar for control and treated hosts, but two of the rarest species, T and Ag, were not detected in disturbed communities. The absence of these rare species is not surprising given that species loss following anthelmintic treatment has been well-documented (Kuzmina and Kharchenko 2008, Craig et al. 2009). It is also possible that the failure to detect T and Ag in treated hosts may have been a function of sample size (9 treated hosts vs. 24 control hosts). Rarefaction suggested that at equivalent host sample sizes, similar numbers of parasite species would have been detected in both treated and control communities. Nevertheless, treated and control communities differed in the contributions of among-host and within-host diversity to overall richness (γ diversity), indicating a fundamental difference in community structure. Species richness in controls was highly variable and spread among hosts rather than within individuals ($\beta > \alpha$ diversity), suggesting an isolationist community with weak interactions among co-occurring parasites (Table 3.1). Second, an isolationist structure was further supported by the shallow-sloped, non-asymptotic species accumulation curve observed for controls. This shape of curve is indicative of low within-host richness and parasites that are distributed randomly among hosts (Dove and Cribb 2006). Conversely, species richness in treated host communities was concentrated within individuals as opposed to among individuals ($\alpha > \beta$ diversity). The a-dominated community and the steep-sloped species accumulation curve are both suggestive of an interactive structure with

high within-host richness and low among-host variability resulting from strong biotic interactions (Dove and Cribb 2006). While total species richness and sample size can influence the shape of species accumulation curves, CC_{50} curves are independent of such differences. Our CC_{50} curves corroborated the species accumulation curves. Treated communities had a steeper slope, and a lower CC_{50} than controls signifying an interactive community structure. All three among-host measures of community structure (α versus β diversity, species accumulation curves, CC_{50}) indicated that control communities were isolationist and disturbed communities were interactive, supporting the prediction that biotic interactions will affect community assembly of non-equilibrium, treated communities, but play a minimal role in equilibrium, control communities.

Parasite communities lie along a spectrum from isolated to interactive, are influenced by environmental conditions, and may vary across populations of the same species and over time (Gotelli and Rohde 2002, Krasnov et al. 2006, Krasnov et al. 2011), so it is not surprising that any two groups of hosts differ in parasite community structure. However, in our study, the control and treated hosts were exposed to the same supply of parasites from the environment, so the differences we observed in community structure indicate differences in host susceptibility to infection after disturbance, rather than differences in exposure. Thus, our artificial disturbance study revealed an important role for biotic interactions in structuring communities during early succession before an equilibrium is reached. Given this, it is possible that individual, population, and species-level differences in time since natural disturbances to the parasite community (e.g. host immunological responses) may explain some of the variability in parasite community structure observed in previous studies.

Notably, at the within-host scale, we found that Cf was the only species more abundant in control hosts than treated hosts. In treated parasite communities, Cf abundance increased as time since treatment increased, suggesting that hosts differentially accumulate Cf compared to other species. This non-random species accumulation pattern lends additional support to the role of biotic interactions in community assembly following disturbance. The predominance of Cf in control communities contributed to lower within-host diversity and evenness, both indicators of an isolationist community structure. Furthermore, as time since disturbance increased, treated communities more closely resembled control communities, indicating the resilience of the equilibrium helminth community. By 8 months post-treatment, treated hosts regained 75% of the mean abundance observed in controls and had nearly identical diversity and evenness indices. Recovery of helminth abundance after anthelmintic disturbance has been documented in many wild populations (Craig et al. 2009, Knowles et al. 2013, Pedersen and Antonovics 2013), but recovery of richness and evenness indices were not considered in these previous studies. Competition-colonization theory predicts that dominant competitors will eventually outcompete other species in the community, erasing differences due to priority effects and the timing of disturbance (Tilman 1994). Our data suggest that biological interactions, most likely competition, are influencing parasite community assembly and structure after disturbance, but play a smaller role in the 'climax', Cf-dominated, control communities.

If Cf is a superior competitor, there are several modes by which it could affect the development and survival of other helminth species: via direct interactions, host immunity, and/or competition for resources. For example, parasites could be competing for space within the GI tract. Although adult Cf primarily inhabit the small intestine (mean = 88% SI) and the other three genera inhabit the abomasum (H: mean = 99% Abo, P: mean = 98% Abo,

Africanastrongylus sp: 100% Abo), larval Cf reside in the lining of the abomasum where they could interact with these other species. Upstream interactions between co-infecting GI parasites are also not unprecedented (Lello et al. 2004), particularly if they are mediated by the host immune system. Experimental infections may provide insight into whether and how these species are competing and reveal additional consequences of community structure for the transmission of these parasites.

While the results above revealed new understanding of parasite community structure and assembly, they were based on lethal sampling. Non-lethal methods offer numerous advantages over lethal methods including the ability to sample large numbers of hosts and to sample individuals repeatedly to track longitudinal changes in parasite communities. Moreover, individuals can be sampled repeatedly to investigate temporal dynamics to understand how parasite communities respond to changes in host physiology (e.g. condition, reproductive status), environmental conditions, and other parasite infections. In our study, non-lethal methods accurately captured patterns of parasite prevalence, overall abundance, and species-specific abundance. Fecal egg count (FEC) were highly correlated with adult worm abundance, and multiplying genetic-based larval composition data by FEC produced reliable estimates of species-specific abundance for Cf. To obtain good species-specific abundance and richness estimates, at least 10 larvae per host are needed. Additionally, community similarity indices using non-lethal sampling data mimicked those based on lethal sampling, establishing non-lethal methods as a viable alternative for studies of parasite community structure.

Conclusions

While equilibrium parasite communities often appear to be randomly assembled, our data suggest that biotic interactions play an important role in the assembly of parasite communities following disturbance. Natural parasite communities may experience a number of different disturbance regimes due to changing host (e.g. stress, reproduction) and environmental conditions (e.g. seasonality, contaminants). Disturbance regimes may vary among host individuals or populations and generate parasite communities in different stages of recolonization at any given time point. Importantly, differences in structure between disturbed and equilibrium communities may account for some of the inconsistencies regarding the role of biotic interactions in shaping parasite communities in wild populations. Thus, to fully understand the factors influencing parasite community structure, it is important to study all phases of community establishment, since equilibrium 'climax' communities may appear isolationist, but biotic interactions among parasites may direct community assembly.

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Table 3.1. Isolationist and interactive communities exhibit contrasting patterns of among- and within-host structure.

	Among-Host Structure			Within-Host Structure			
	Distribution of Richness	Species Accum. Curve	CC_{50}	Abundance	Richness	Diversity	Evenness
Isolationist	high variability ($\beta > \alpha$ diversity)	shallow slope	high	low	low	low	low
Interactive	low variability ($\alpha > \beta$ diversity)	steep slope	low	high	high	high	high

Figure Legends

Figure 3.1. Relative abundance of helminth species pooled across anthelmintic-treated (n =9) and control hosts (n = 22).

Figure 3.2. Jackknife estimates (± 1sd) of species accumulation curves by host (a) and by individual worm (b). Cumulative species richness and the percentage of treated (T) and control (C) hosts needed to accumulate 50% of total richness (CC_{50}) are shown in (c). The jackknife species accumulation curve (± 1sd) for non-lethal larval sampling (d) followed a similar trend.

Figure 3.3. Community composition metrics (±1 se), for control and treated hosts including: (a) total abundance of adult worms, (b) species richness, (c) Simpson diversity index, and (d) evenness. Asterisks (*) indicates significant differences ($p < 0.05$).

Figure 3.4. Mean within-host abundance of each helminth species in control and anthelmintic treated hosts (±1 se). Asterisks (*) indicates significant differences between treatments ($p <0.05$).

Figure 3.5. Relationship between the capture interval (number of days since anthelmintic treatment) and (a) total abundance of adult worms, (b) species richness, (c) Simpson diversity index, (d) evenness, and (e) species-specific abundance. Solid lines indicate significant correlations and dashed lines indicate non-significant correlations. Horizontal black lines (a-d) indicate the mean values for control hosts.

Figure 3.6. The difference between lethal and sub-lethal estimates of the relative abundance of species (Cf = H) is shown in (a). Across a sample of 127 hosts, (b) within-host richness (±1 sd) increased and (c) the mean number of species missed decreased with the number of larvae identified per host. In (d), the mean difference between observed and estimated proportional abundance for each species was near zero (horizontal gray dashed line) across all subsample sizes, but variability (95% CI) decreased as the number of larvae identified per host increased.

Figure 3.7. Lethal, adult-based estimates of abundance and sub-lethal fecal egg counts (FEC) were significantly correlated. Correlations between adult-based and sub-lethal, larvae-based estimates of species-specific proportional abundance for (b) Cf and (c) H. The size of points reflects the number of larvae identified per host and the gray square indicates the one bolus individual (b and c).

Suppl. Fig. 3.1. Comparison of component community composition between lethal, adult-based sampling and sub-lethal, larvae-based sampling.

Figures

Figure 3.1

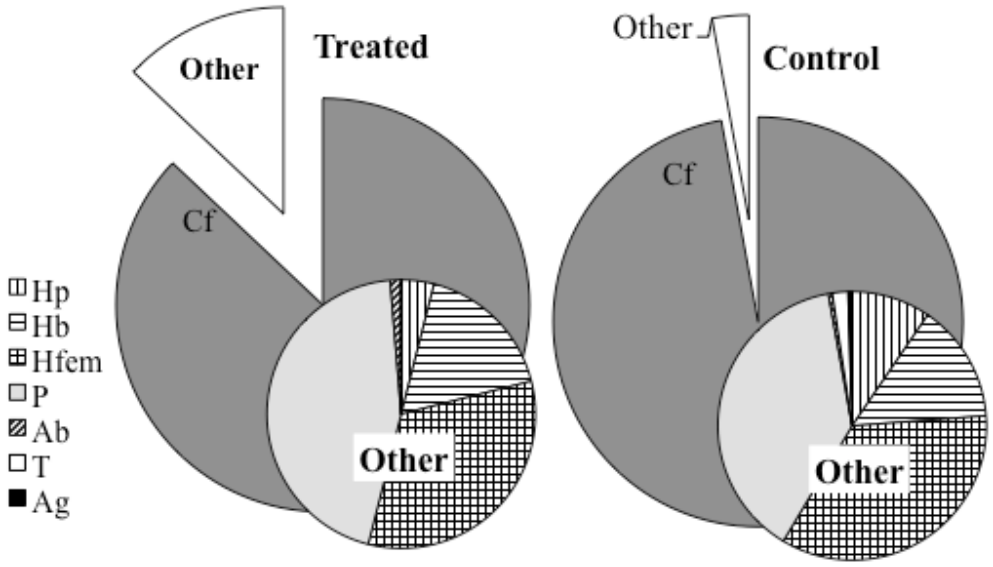


Figure 3.2

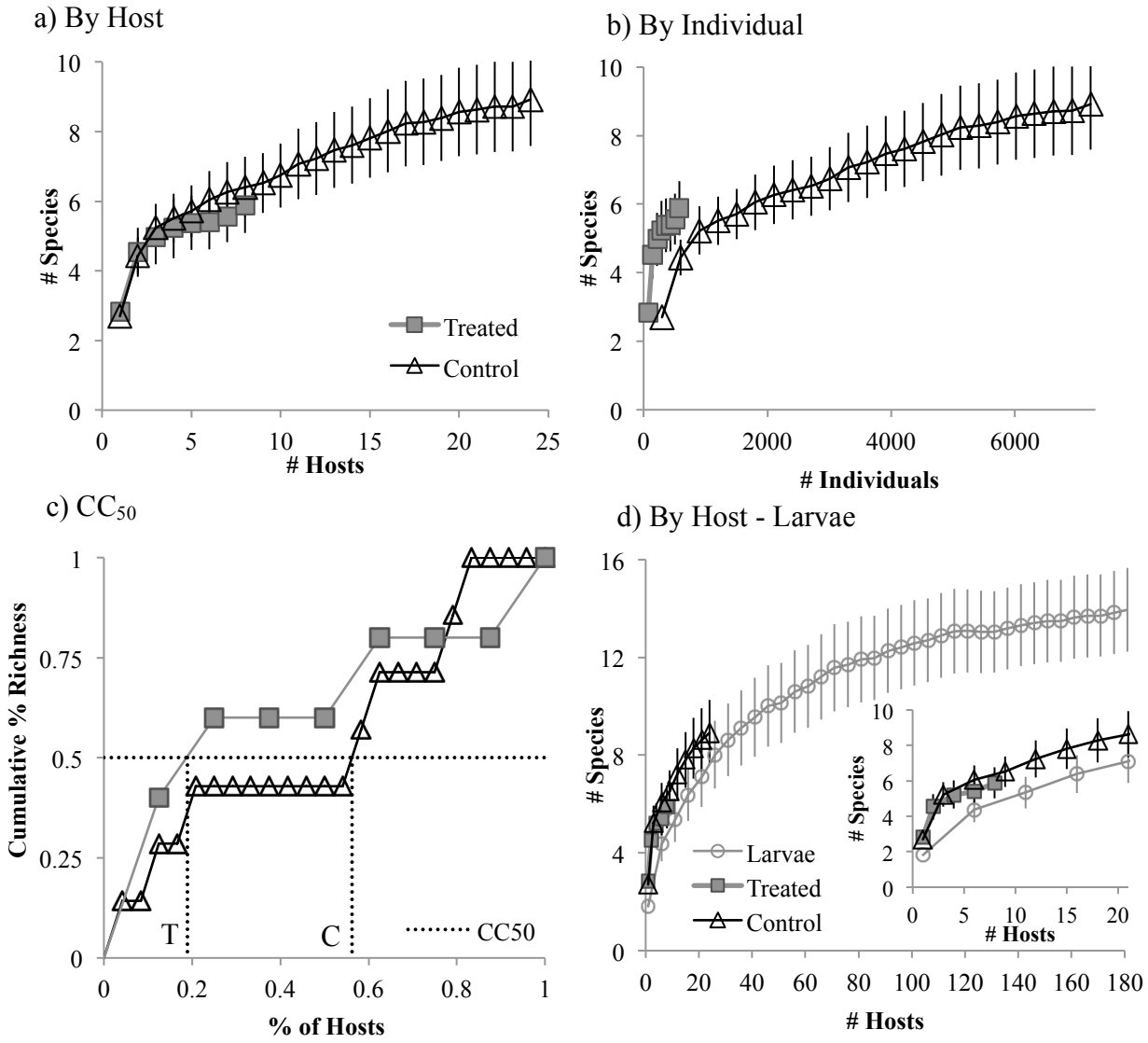

a) By Host
Species
Hosts
Treated
Control
b) By Individual
Species
Individuals
c) CC50
Cumulative % Richness
% of Hosts
T
C
CC50
d) By Host - Larvae
Species
Hosts
Larvae
Treated
Control

Figure 3.3

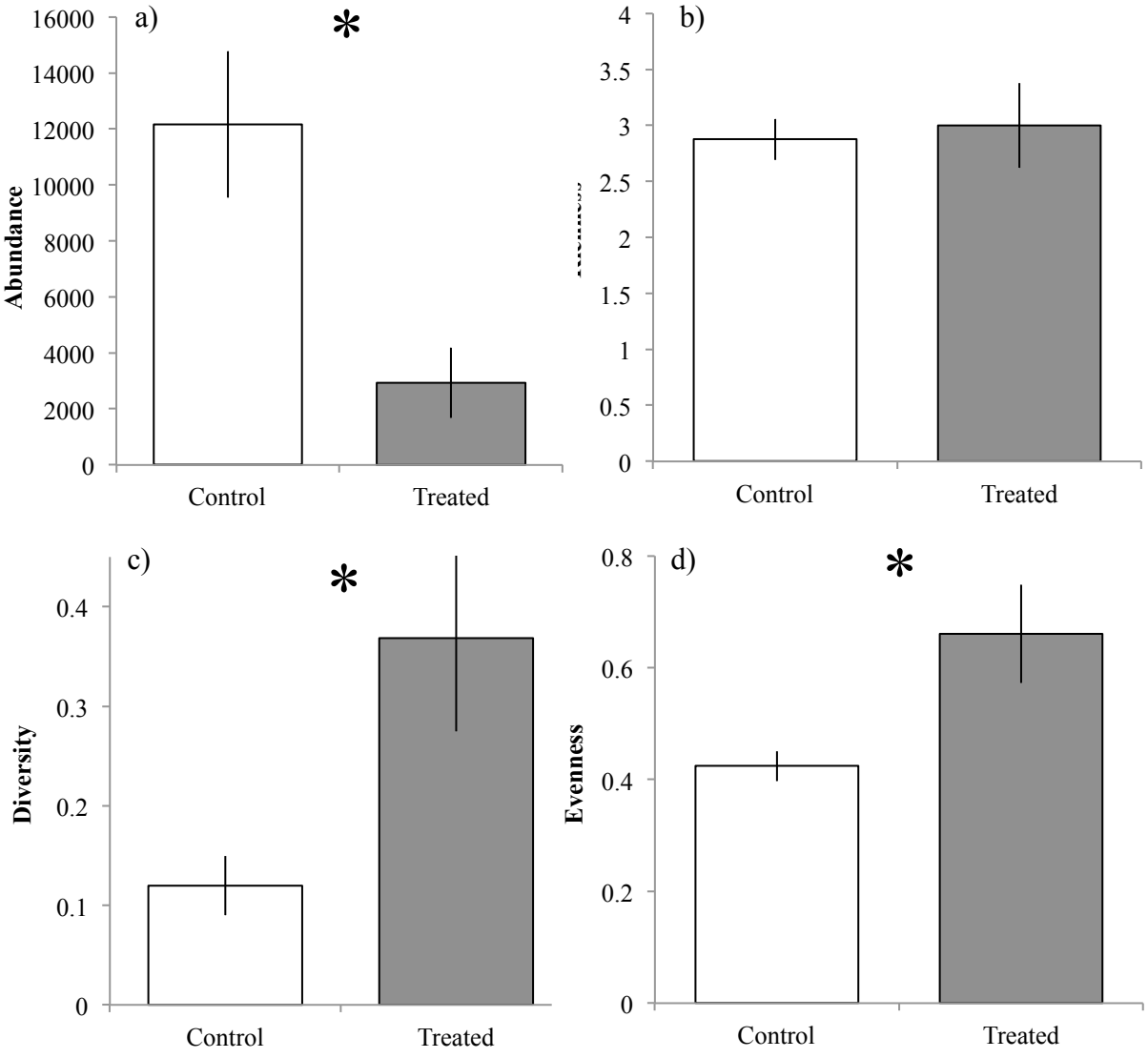
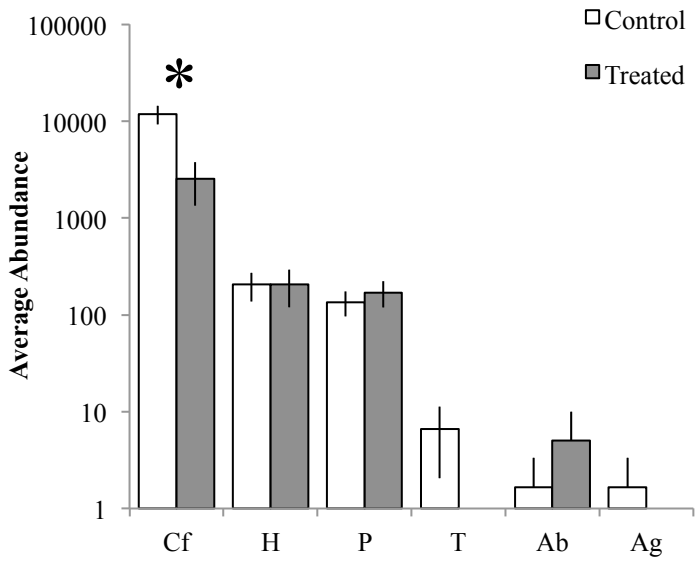

a)
*
Abundance
16000
14000
12000
10000
8000
6000
4000
2000
0
Control
Treated
b)
4
3.5
3
2.5
2
1.5
1
0.5
0
Control
Treated
c)
*
Diversity
0.4
0.3
0.2
0.1
0
Control
Treated
d)
*
Evenness
0.8
0.6
0.4
0.2
0
Control
Treated

Figure 3.4


Control
Treated
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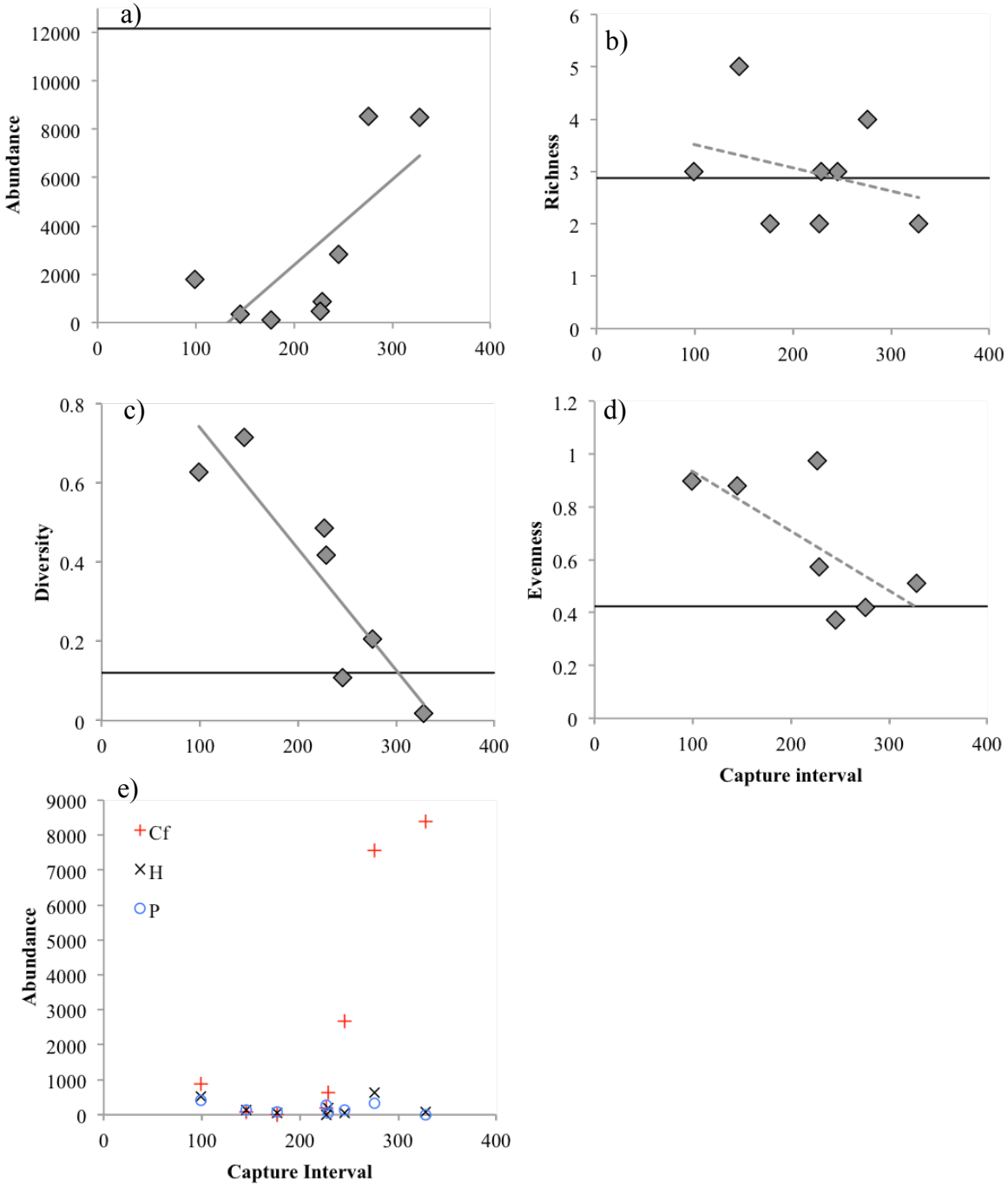
10000
1000
100
10
1
Average Abundance
*
Cf
H
P
T
Ab
Ag

Figure 3.5


a)
Abundance
12000
10000
8000
6000
4000
2000
0
0
100
200
300
400
b)
Richness
6
5
4
3
2
1
0
c)
Diversity
0.8
0.6
0.4
0.2
d)
Evenness
1.2
1
0.8
0.6
0.4
0.2
Capture interval
e)
Cf
H
P
Abundance
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8000
7000
6000
5000
4000
3000
2000
1000
Capture Interval

Figure 3.6

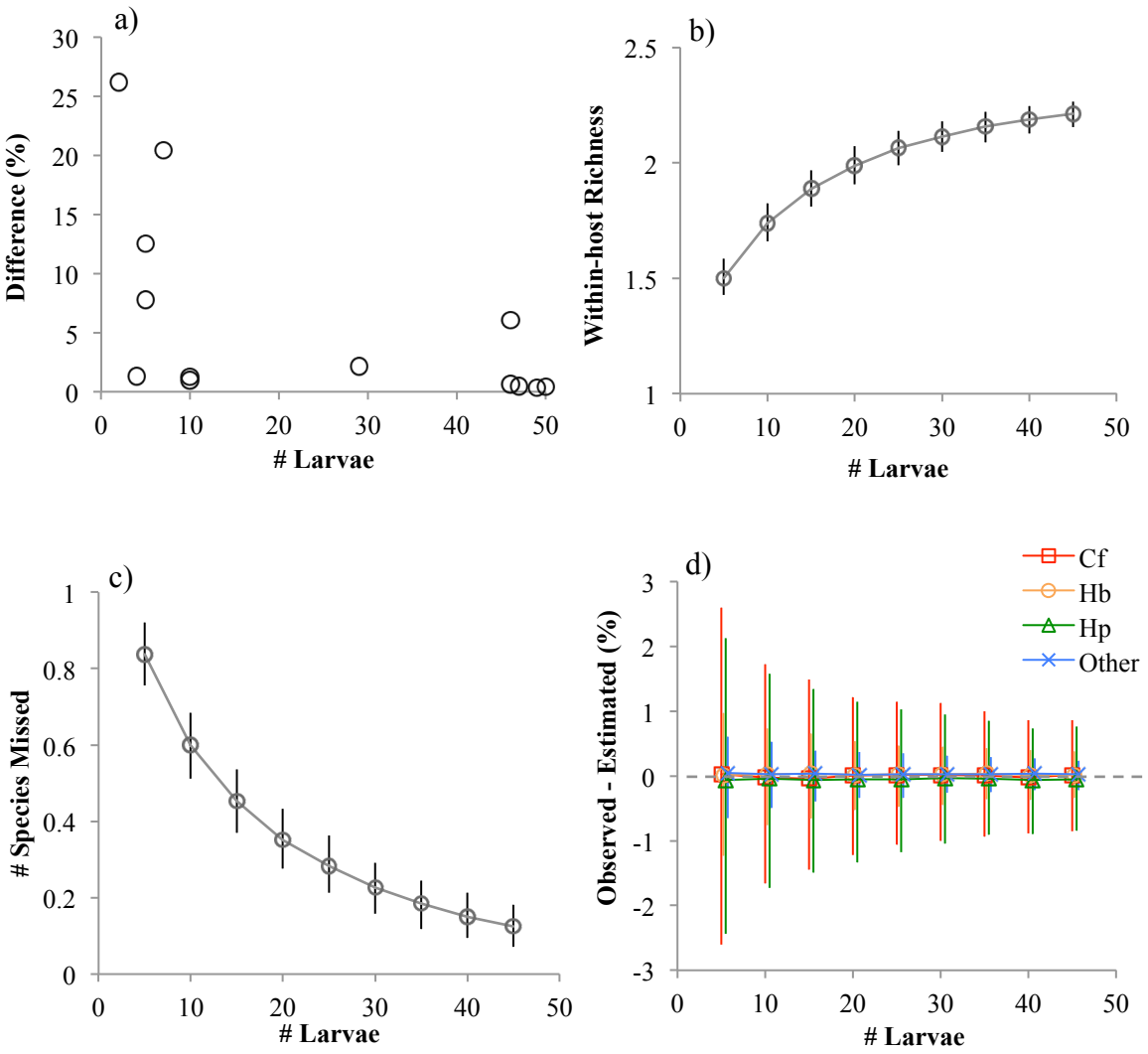
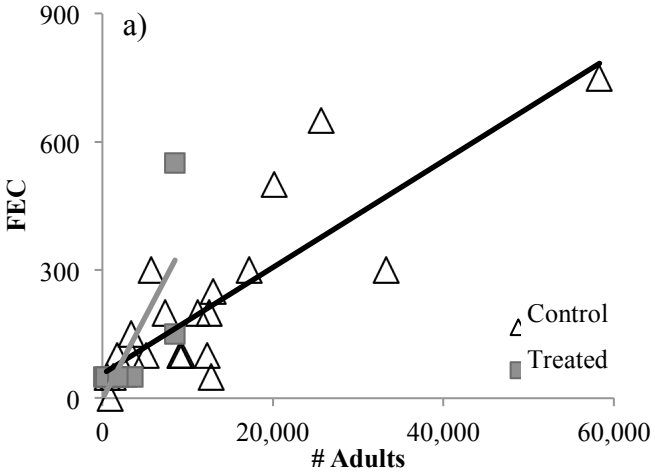

a)
Difference (%)
Larvae
b)
Within-host Richness
Larvae
c)
Species Missed
Larvae
d)
Observed - Estimated (%)
Larvae
Cf
Hb
Hp
Other

Figure 3.7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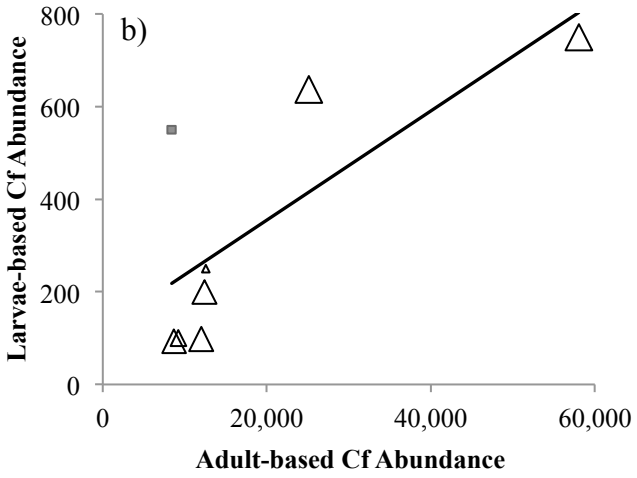
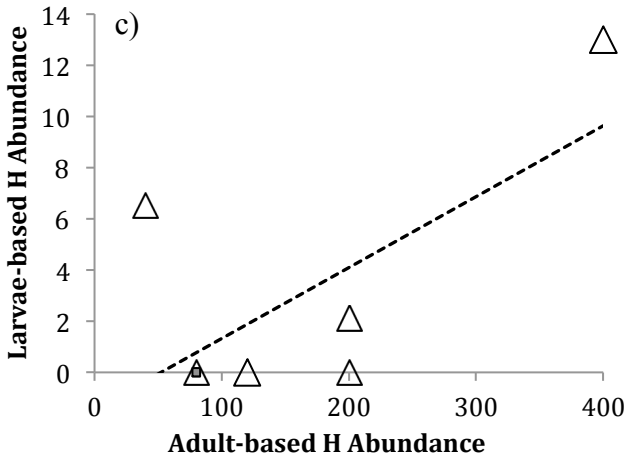
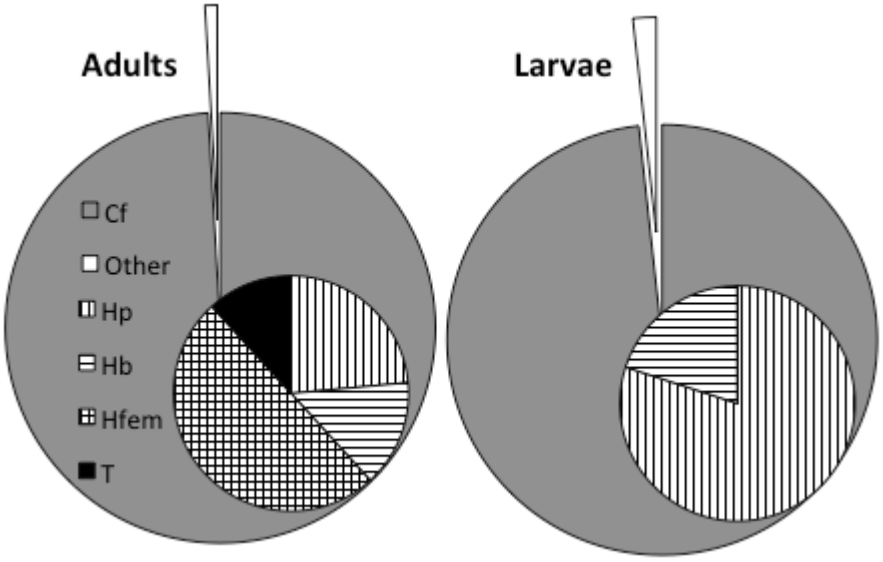
300
0
FEC
0
20,000
40,000
60,000
Adults
Control
Treated


b)
800
600
400
200
0
Larvae-based Cf Abundance
0
20,000
40,000
60,000
Adult-based Cf Abundance


c)
14
12
10
8
6
4
2
0
Larvae-based H Abundance
0
100
200
300
400
Adult-based H Abundance

Suppl. Fig. 3.1



CHAPTER 4

RESOURCE LIMITATION ALTERS THE CONSEQUENCES OF COINFECTION FOR BOTH HOSTS AND PARASITES[1]

[1] Budischak, S. A., K. Sakamoto, L. C. Megow, K. Cummings, J.F. Urban, Jr., and V. O. Ezenwa.

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Abstract

Most animals are concurrently infected with multiple parasite species and live in environments with fluctuating resource availability. Resource limitation can influence host immune responses and the degree of competition between co-infecting parasites, yet its effects on individual health and pathogen transmission have not been studied for co-infected hosts. To test how resource limitation and immune function affect the outcomes of co-infection, we conducted a factorial experiment using laboratory mice. Mice were given a standard or low quality diet, dosed with two species of helminths (alone and in combination), and then challenged with a microparasite. Co-infection influenced host immune function and parasite survival and reproduction, but the magnitude and direction of responses depended on diet and the combination of co-infecting parasites. Facilitative interactions between the two helminth species were weaker among mice fed a low protein diet, while facilitative interactions between helminths and the microparasite were stronger. Observed effects of co-infection and resource limitation on parasite reproduction were indirect and immune-mediated rather than direct and resource-mediated. Our findings highlight that resources and their consequence for host defense are a key context that shapes the magnitude and direction of parasite interactions.

Introduction

Most free-living animals are infected with multiple parasite species simultaneously with coinfection being the norm rather than the exception (Petney and Andrews 1998). These parasites can be from similar taxonomic and functional guilds, or as distinct as viruses and parasitic worms Co-infection can affect host susceptibility to future infections (Telfer et al. 2010), realized parasite virulence (May and Nowak 1995), and a number of other host and

parasite traits. In addition to being challenged by multiple parasites, hosts often live in environments where resource availability varies spatially and temporally. Resource limitation can affect host immune defenses against parasites (Koski and Scott 2001), and many co-infecting parasites interact indirectly via the host immune system (Cox 2001). Interactions between parasites within hosts may also be mediated by competition for shared resources (Graham 2008). Yet, despite the considerable potential for resources to influence both immune- and resource-mediated interactions among co-occurring parasites, the effects of host resources on host and parasite performance (e.g. growth, fecundity, etc.) during co-infection are largely undescribed.

Ecological theory offers a mechanistic framework for understanding the potential network of direct and indirect interactions that can occur among hosts and parasites (Pedersen and Fenton 2007). When a classic trophic framework is applied to parasites, the host's immune defenses are analogous to top-down, predation pressure, whereas host resources exert bottom-up effects on parasites by limiting critical nutrients (Graham 2008). Notably, indirect interactions between parasites and host immunity also arise because host immune responses often depend on underlying resource availability (French et al. 2009). Therefore, the effect of resource augmentation on the fitness of any single parasite can be positive or negative depending on whether added host resources are used by parasites for replication (direct effect) or by hosts for immune defense (indirect effect) (Cressler et al. 2014). With this in mind, the consequences of added resources for the outcome of co-infections can be challenging to predict, because positive and negative effects can arise depending on whether co-occurring parasites compete for the same resources, and whether the effects of immune interactions are antagonistic or facilitative.

To investigate how resources and immunity influence interactions among co-occurring parasites, with resulting effects on host and parasite fitness, we conducted a co-infection

experiment using laboratory mice (*Mus musculus*). Mice were fed either a standard or low protein diet, dosed with one or two species of parasitic nematodes, *Nippostrongylus brasiliensis* (Travassos; *Nb*) and *Heligmosomoides polygyrus bakeri* (Dujardin; *Hb*), and then challenged with an intracellular microparasite, *Mycobacterium bovis (Mb).* Adult *N. brasiliensis* and *H. bakeri* worms live in the small intestine, although *N. brasiliensis* larvae first migrate through the lungs. *N. brasiliensis* stimulates a T-helper type 2 (Th2) immune response, whereas *H. bakeri* triggers a regulatory T cell (Treg) response (Rausch et al. 2008, Maizels et al. 2012). The microparasite, *M. bovis*, stimulates a T-helper type 1 (Th1) immune response during primary infection, which occurs in the lungs (Flynn and Chan 2001). T-helper lymphocytes coordinate immune responses by secreting chemical messengers (cytokines) to direct the action of other immune cells. Th1 and Th2 immune responses are mutually inhibitory, and the cytokines associated with each response down-regulate the other, which ultimately leads to facilitative interactions between helminths and intracellular microparasites (Maizels et al. 2004). Importantly, the regulatory (Treg) response stimulated by *H. bakeri* suppress both Th1 and Th2 immunity, which can lead to facilitative interactions between helminths and a wide range of other parasites, including other worms (Maizels et al. 2004). As such, all three of our focal parasites could interact via the host immune system. In addition, *H. bakeri* and *N. brasiliensis* consume similar nutrients in the host intestine, so direct resource competition between these two species is also possible.

Using this model of three parasites with different immune phenotypes, we tested a series of non-exclusive predictions about how resource availability and immunity combine to influence parasite interactions. In the presence of a nutritionally complete diet, we expected that the Treg response stimulated by *H. bakeri* infection would reduce Th2 responses to *N. brasiliensis* and

positively affect *N. brasiliensis* egg shedding. We also predicted hosts to respond to microparasite infection with a strong Th1 response that could reduce immune defense to the helminths and increase egg shedding. Further, we predicted that resource limitation (i.e. low protein diet) could affect pairwise interactions between the parasites in several ways. (1) For the two helminth parasites, limited diet might relax Treg-Th2 facilitation, with a net negative effect on *N. brasiliensis* but no effect on *H. bakeri*; alternatively, limited diet might intensify resource competition, with a net negative effect on either *N. brasiliensis* or *H. bakeri* or both. (2) In terms of interactions between the microparasite and helminths, limited diet might relax or intensify either Th1-Th2 facilitation and/or Th1-Treg facilitation. Thus, the outcome could cause a net positive effect or a net negative effect on *N. brasiliensis*, with a lesser effect on *H. bakeri*. We also expected that limited host resources and any interactions that increased parasite fitness would ultimately decrease host performance. To fully understand this complex network of interactions, we combined structural equation models with more traditional analyses to quantify the direction and strength of connections among parasites, resources, and immunity.

Materials and methods

Animal and diet protocols

We used a factorial experimental design with two diet treatments (standard vs. low protein), four helminth treatments (no nematodes, *H. bakeri* only, *N. brasiliensis* only, and both nematodes together), and two *M. bovis* treatments (no *Mb* or *Mb*) to investigate the consequences of co-infection. Like individuals in wild populations, genetic lines of laboratory mice vary in their predisposition for Th1 versus Th2 responses. We selected a line of mice with robust Th2 responses (BALB/c) to test how coinfection and diet influenced that top-down pressure on

helminth reproduction. We randomly assigned eight mice (Harlan Laboratories Inc.) to each treatment combination, and mice were housed four per cage. All mice were female and 6-7 weeks old (representing reproductively mature adults) at the beginning of the experiment.

Prior to the start of the experiment, all mice were fed a standard protein (SP) rodent diet (LabDiet® 5002, 21% protein), and at the start of the experiment (Day 0 [D0]) half of the mice were switched to a low protein (LP) diet (LabDiet® 5CR4, 14% protein). Both diets have nearly identical caloric content and micronutrient composition. Protein malnutrition is strongly associated with increased susceptibility to many parasites and pathogens (Ing et al. 2000, Koski and Scott 2001), but it is unclear whether these effects are mediated by changes in host immune function, parasite resource limitation, or both. Owing to the intense demands of implementing the experiment and data collection, we ran the entire experiment for the standard protein diet group first, then repeated it for the low diet group. Within each diet replicate, we also staggered the start day of each helminth treatment over the course of five days. Mice were fed *ad libitum* and weighed to the nearest 0.1 g at the beginning of the experiment (D0), one week after initiation of the diet treatment (D6), and every second day thereafter.

Helminth infection

Mice received helminth treatments one week after the start of the experiment (D6) since this period was sufficient to establish diet-based differences a single-infection protein limitation study (Tu et al. 2007). Mice infected with only *Heligmosomoides p. bakeri* (hereafter called HB) and coinfected both nematodes (hereafter called COINF) were intubated orally with 200 infective *H. p. bakeri* larvae. Coinfected (COINF) mice and those infected with only *Nippostrongylus brasiliensis* (hereafter called NB), received 200 infective *N. brasiliensis* larvae

via subcutaneous injection. Mice with no nematodes (hereafter referred to as CTL) received equal volumes of sterile PBS via oral gavage and subcutaneous injection. On D14, eight mice per treatment (representing one third of the mice in total, none of which had been infected with the microparasite) were euthanized to examine host immune responses. D14 is an important time-point because it falls after both helminths complete development, but prior to clearance of *N. brasiliensis*. Adult helminth counts were not determined because intestinal tissues from euthanized mice were immediately processed for flow-cytometric immune assays that required live cells. To quantify helminth fecundity, individual mice were isolated in separate cages for 30-120 minutes every second day from D6-D28 for fecal sample collection. The number of helminth eggs per gram of feces was counted using a modified McMaster egg fecal counting protocol (Ministry of Agriculture and Food 1980). Based on preliminary single infection trials, the eggs of the two helminth species were distinguished based on size and color (Fig S4.1).

Microparasite infection

Mycobacterium bovis infection was initiated eight days post-helminth infection on D14. Eight mice per helminth and diet treatment (n = 64 mice in total) were infected intratracheally with 60 colony forming units (CFU) of *M. bovis* (hereafter called MB+), with the control mice referred to as MB- (n = 64 mice). Mice were euthanized two weeks after *M. bovis* infection (D28) to examine how diet and helminth community composition influenced *M. bovis* infection. PCR was used to verify the presence of *M. bovis* in the inoculum and to confirm infection in lung tissue homogenates from a subsample of MB+ mice (Parsons et al. 2002).

Immune assays

To assess immune functions that are known to be relevant to helminth infection, we quantified eosinophil responses. Eosinophils are one of the main white blood cell types upregulated by the Th2 response and are elevated during most helminth infections (Janeway 2008). We quantified eosinophils in two ways: by histopathology, which provides a qualitative assessment of eosinophil abundance and location, and by flow cytometry, which provides a more quantitative estimate of the proportion of cells in the tissue that are eosinophils. We also attempted to quantify Th1 and Th2 cytokine concentrations from peripheral blood samples and ex vivo cytokine production of lung and intestine tissues. Unfortunately, circulating cytokine concentrations fell below detection limits and ex vivo tissue samples varied in size and cell viability, so we were unable to accurately assess host investment in Th1 defenses.

For histopathology, a 0.5 cm^2 piece of lung tissue and 1 cm piece of the proximal end of the small intestine were collected from each mouse. Intestinal tissue sections were scored for the number of eosinophils infiltrating the lamina propria on a scale of 0 to 4 (Table S4.1). For lung tissue, the number of eosinophils cuffing medium-caliber blood vessels was scored on a scale of 0 to 4 (Table S4.1). For flow cytometry, cells were isolated from homogenized small intestine and lung tissues. Isolated cells were stained with fluorescent markers for viability (LIVE/DEAD® Fixable Violet Stain Kit, Invitrogen) and eosinophil surface proteins (APC anti-MHCII, PE anti-Siglec-F, and FITC anti-CD11), then fixed in formalin. Fluorescence was measured using a CyAn ADP Analyzer™ (Beckman Coulter), and eosinophils were quantified by calculating the percentage of single, live cells that were MHCII negative, Siglec-F positive, and CD11 intermediate (Stevens et al. 2007).

Statistical Analysis

Helminth Egg Shedding

We calculated total egg shedding for each helminth species by integrating under the fecal egg count vs. time curve for each mouse. We used general linear models (GLM) to test the effects of diet, helminth infection, *M. bovis* infection and their interactions on total helminth egg shedding over the course of infection for both helminth species. When necessary, data were box-cox transformed so that model residuals were normally distributed. Tukey's post-hoc tests were used to compare between treatments when significant effects were detected. *N. brasiliensis* infection duration was analyzed using a similar procedure. *H. bakeri* infection duration was not examined since most mice remained infected at the end of the experiment on D28.

For each treatment group, we assessed the likelihood that individuals were super-shedders (i.e. individuals that disproportionately to transmission). Based on the distribution of total egg shedding for each helminth species, individuals falling in the upper 20% were considered super-shedders (Lass et al. 2013). For each helminth species, we used a Chi-square test to determine if there were more super-shedders than expected due to diet, helminth co-infection, or *M. bovis* infection.

Mouse weight

We examined the effect of diet, helminth infection, and *M. bovis* infection on mouse weight using single and multi-factor ANOVA. First, we examined the effect of diet during the first week of the experiment by testing for differences in weight gain (or loss) between D0 and D6 using mice euthanized prior to helminth infection. Second, we tested for effects of diet and helminth treatment on weight gain between the start of the experiment and D14 using mice

euthanized on D14. Finally, we examined how diet, helminth infection, and *M. bovis* infection interacted to influence weight gain between D14 (*M. bovis* infection time point) and D28 (end of experiment). Tukey's post-hoc tests were used to test for differences among treatment combinations when main effects were significant.

Immunity

Separate analyses were run for histopathological versus flow cytometry-based immune measures, as well as for responses from the intestine versus the lung at different time points (D6, D14, and D28). Eosinophil scores derived from histopathology were analyzed using Kruskal-Wallis (KW) tests since the rank-based scores were not normally distributed. For these analyses we only considered the main effects of diet, helminth infection, and *M. bovis*. Kruskal-Wallis post-hoc tests were used to compare among helminth treatments. For the eosinophil measures derived from flow cytometry, we used GLMs that included the main effects and their 2-way interactions, followed by Tukey's tests to compare among helminth treatments. In the one case where the data were not normally distributed (D14, intestinal eosinophil levels), we used a KW test to determine the effects of diet and helminth treatments.

Structural equation models

In addition to the traditional analyses above, we used structural equation models (SEM) to examine the relative importance of direct and indirect effects on helminth egg shedding. SEM provides a means of simultaneously testing the relative importance of multiple directional paths (Grace 2006). So that *M. bovis* co-infection and total helminth egg shedding data could be included in the model, only data from animals sampled on D28 were included. We used the

lavaan package in R to test the overall fit of the SEM models (X^2, Akaike information criterion [AIC]) (Rosseel 2012), and evaluated the strengths of relationships between variables using standardized coefficients. We used information from previous research and our own results to hypothesize causal linkages for models of *N. brasiliensis* egg shedding (for NB and COINF mice) and *H. bakeri* egg shedding (for HB and COINF mice) (Fig 4.1A). We explicitly tested whether: 1) co-infecting parasites influence both immunity and resources, 2) diet influences immunity and resources, 3) immunity influences resources or, conversely, resources influence immunity, and 4) egg shedding influences and is influenced by immunity and resources. For these analyses, lung and intestinal eosinophil percentages from flow cytometry were log transformed to improve normality. We modified the original models by testing for missing links based on model residuals and by dropping poorly supported links until the change in AIC was less than 2 (Grace 2006).

Results

Resource Limitation: Effect on Parasites

The two helminth species showed distinct patterns of egg shedding, and resource limitation affected these patterns differently. *N. brasiliensis* egg shedding began between 4 and 6 days post infection (D10-D12; Fig. 4.2A), whereas *H. bakeri* egg shedding began 8 days post infection (D14) and remained high throughout the duration of the experiment (Fig. 4.2B). Both mean and maximum *H. bakeri* egg shedding were over 20-fold higher than *N. brasiliensis* egg shedding (mean: *Hb* = 43361 ± 3777 epg, *Nb* = 1217 ± 214 epg, maximum: *Hb* = 139333 epg, *Nb* = 6657 epg). Dietary protein did not affect the duration of *N. brasiliensis* egg shedding ($F_{1,57}$ = 0.05, p = 0.83; Fig. 4.2A), but there was a significant effect of diet on the total number of *N.*

brasiliensis eggs shed over the course of the experiment. Mice fed the standard protein diet shed more *N. brasiliensis* eggs than those on the low diet ($F_{1,57} = 21.0$, $p < 0.0001$; Fig. 4.2A, inset). In contrast, mice fed the standard protein diet shed fewer *H. bakeri* eggs than those on the low diet ($F_{1,57} = 7.18$, $p = 0.010$; Fig. 4.2B, inset). Interestingly, mice fed the standard protein diet were more likely to be *N. brasiliensis* super-shedders than mice fed the low diet (SP = 31%, LP = 9.4%, $X^2_1 = 4.73$, $p = 0.03$), but diet did not influence *H. bakeri* super-shedding ($X^2_1 = 2.41$, $p = 0.12$).

Resource Limitation: Effects on Host

Resource limitation also had effects on the host. Prior to helminth infection (D0-D6), mice fed the standard protein diet gained over twice as much weight as those fed the low diet ($F_{1,14} = 5.0$, $p = 0.041$; Fig. 4.3A). However, diet had no detectable effect on mouse weight gain for the remainder of the experiment (D14: $F_{1,56} = 0.01$, $p = 0.91$; D28: $F_{1,115} = 0.17$, $p = 0.16$). Diet interacted with total *N. brasiliensis* egg shedding to influence weight gain. There was no association between *N. brasiliensis* egg shedding and weight gain for HP mice, whereas low mice gained less weight for a given egg burden ($F_{1,60} = 5.7$, $p = 0.02$; Fig. 4.4A). Conversely, diet did not influence the relationship between *H. bakeri* infection and weight gain ($F_{1,60} = 1.8$, $p = 0.18$; Fig. 4.4B).

Intestinal immunity was affected by resource limitation across multiple time points. On D6, mice fed the low diet had higher histopathological eosinophil scores than those fed the standard protein diet (LP: 2 ± 0, SP: 1.38 ± 0.28; $X^2_1 = 6.82$, $p = 0.009$), but there was no effect of diet on eosinophil levels measured by flow cytometry (LP: 0.22 ± 0.10, SP: 0.26 ± 0.13; $F_{1,14} = 0.02$, $p = 0.88$). Histopathological eosinophil scores remained higher among mice fed the low

diet on D14 (LP: 1.97 ± 0.11, SP: 1.56 ± 0.12) and D28 (LP: 2.48 ± 0.11, SP: 1.89 ± 0.12; Table 5.1; Fig. S4.2). At these later time points, the flow cytometry data supported the pattern found with the histopathological scores; mice fed the low diet had higher percentages of eosinophils in the intestine on both D14 (LP: 1.99 ± 0.29, SP: 1.40 ± 0.38) and D28 (LP: 1.32 ± 0.14, SP: 0.62 ± 0.10; Table 4.2; Fig. S4.3).

The effects of diet on immune responses in the lung were less pervasive. No eosinophils were observed by histopathology in the lungs of mice on D6 prior to the start of infection. While eosinophil levels measured by flow cytometry varied from 0-10% on D6, there was no detectable effect of diet on these values (LP: 4.95 ± 1.22, SP: 5.58 ± 0.45; $F_{1,14} = 0.24$, $p = 0.63$). Histopathological eosinophil scores from the lung did not differ by diet on either D14 (LP: 1.38 ± 0.28, SP: 1.19 ± 0.24) or D28 (LP: 0.61 ± 0.12, SP: 0.55 ± 0.11; Table 4.1; Fig. S4.2). However, flow cytometry data showed that the percentage of eosinophils in the lungs was significantly higher in mice fed the low diet on both D14 (LP: 9.96 ± 1.16, SP: 7.28 ± 0.67) and D28 (LP: 12.33 ± 0.86, SP: 7.77 ± 0.76; Table 4.2; Fig. S4.3).

Helminth co-infection: Effect on Parasites

Co-infection had strong effects on parasites, but these effects were asymmetrical. For *N. brasiliensis,* the duration of egg shedding, the total number of eggs shed, and variability in shedding were all altered by co-infection. COINF mice shed *N. brasiliensis* eggs for 12.1 days post infection compared to 7.4 days for NB mice, and this difference in infection duration was significant ($F_{1,57} = 146$, $p < 0.0001$; Fig. 4.2A). There was also a significant interaction between diet and helminth treatment; low diet led to an 1.8 d longer *N. brasiliensis* infection duration for COINF mice, but a 0.7 d shorter duration for NB mice ($F_{1,57} = 8.57$, $p = 0.005$). With respect to

total egg shedding, COINF mice shed almost 7-fold more *N. brasiliensis* eggs than did NB mice ($F_{1,57} = 108$, $p < 0.0001$; Fig. 4.2A). Notably, COINF mice were also more likely to be *N. brasiliensis* super-shedders compared to singly-infected NB mice (COINF: 68%, NB: 0%; X^2_1 16.3, $p < 0.0001$). Co-infection did not influence total number of *H. bakeri* eggs shed or variability in *H. bakeri* egg shedding. Total *H. bakeri* egg shedding did not differ between HB and COINF mice ($F_{1,57} = 0.15$, $p = 0.70$), and there was no interaction with diet ($F_{1,57} = 0.07$, $p = 0.79$). Likewise, there was no effect of co-infection on the occurrence of *H. bakeri* super-shedding (HB: 33%; COINF: 18.5%; $X^2_1 = 0.873$, $p = 0.35$).

Helminth co-infection: Effects on Host

Infection with individual helminth species had demonstrable effects on host weight (D14: $F_{3,56} = 4.93$, $p < 0.01$; D28: $F_{3,115} = 5.61$, $p = 0.001$), and co-infected mice experienced similar reductions in weight gain as singly-infected mice. Between D0 and D14 when mice were shedding *N. brasiliensis*, but not *H. bakeri* eggs, both NB and COINF mice gained 70% less weight than HB mice, driving a significant difference in weight gain among these groups ($p < 0.025$; Fig. 4.3B). Both NB and COINF mice also gained 51% less than CTL mice, although this difference was not significant (Fig. 4.3B, $p < 0.15$). Between D14 and D28, HB mice gained significantly less weight than CTL and NB mice (HB-NB: $p < 0.001$, HB-CTL: $p < 0.025$; Fig. 4.3C), which corresponds to the time period when mice were shedding *H. bakeri* eggs.

Overall, helminth infection had significant effects on both intestinal and lung immunity (Tables 4.1, 4.2), but once again, there was little evidence of an added co-infection effect. With respect to intestinal immunity, CTL mice consistently had lower eosinophil responses than helminth-infected mice, and co-infected mice never had higher responses than singly-infected

mice. On D14, histopathological eosinophil scores were elevated in NB mice compared to CTL and COINF mice, and on D28, scores were higher in both singly-infected treatments, NB and HB, compared to CTL mice ($p < 0.05$; Fig. S4.2). Additional differences among treatments were detected using flow cytometry data. On D14, all helminth-infected mice (NB, HB, and COINF) had higher intestinal eosinophil levels than CTL mice ($p < 0.05$; Fig. S4.3). Similarly, on D28, flow cytometry data revealed that eosinophil levels remained elevated in the intestines of all infected mice compared to controls (HB-CTL, COINF-CTL: $p < 0.001$, NB-CTL: $p < 0.05$; Fig. S4.3). Interestingly though, COINF mice had higher intestinal eosinophil levels than NB mice ($p < 0.05$; Fig. S4.3).

In the lungs, eosinophils as measured by histopathology were elevated in NB mice and remained high throughout the experiment (Table 4.1), likely because infective *N. brasiliensis* larvae molt into L4 larvae in the lung, leaving behind an immunogenic cuticle. Specifically, histopathological eosinophil scores in the lung were elevated in NB and COINF mice on both D14 and D28, long after the migration of *N. brasiliensis* larvae through the lung and clearance of *N. brasiliensis* adults ($p < 0.05$; Fig. S4.2). Flow cytometry data showed less clear patterns. On D14, COINF mice had higher lung eosinophil levels than CTL, NB, and HB mice, but only when fed the low diet (COINF-CTL $p < 0.001$, COINF-HB, COINF-NB: $p < 0.01$; Fig. S4.3), accounting for a significant interaction effect of diet and helminth infection on lung immunity (Table 4.2). On D28, eosinophil levels were elevated in the lungs of HB mice compared to CTL and NB mice ($p < 0.025$).

Helminth-M. bovis co-infection: Effect on Parasites

Similar to helminth co-infection, helminth-*M. bovis* co-infection had clear effects on parasites, and these effects were strongly asymmetrical and dependent on diet. *M. bovis* infection had no main effect on *N. brasiliensis* infection duration ($F_{1,57} = 1.48$, $p = 0.23$), but there was a significant interaction between diet and *M. bovis* ($F_{1,57} = 7.15$, $p = 0.010$), such that MB+ mice on the low diet shed *N. brasiliensis* eggs for 1.8 days longer than MB- mice of the same diet ($p = 0.039$). Likewise, there was no main effect of *M. bovis* infection on total *N. brasiliensis* egg shedding ($F_{1,57} = 1.38$, $p = 0.25$), but diet interacted with *M. bovis* ($F_{1,57} = 10.7$, $p = 0.002$), such that MB+ mice on the low diet had 3.5-fold higher egg shedding than MB- mice ($p = 0.014$; Fig. 4.5). For mice fed the standard protein diet, there was no difference in egg shedding between MB+ and MB- mice ($p = 0.40$, Fig. 4.5). Thus, *M. bovis* infection had a positive effect on *N. brasiliensis*, but only when host resources were limited. *M. bovis* infection did not affect the likelihood of being a *N. brasiliensis* super-shedder (MB-: 23%, MB+: 28%; $X^2_1 = 0.10$, $p = 0.75$). *M. bovis* infection did not influence total *H. bakeri* egg shedding ($F_{1,57} = 0.32$, $p = 0.57$). There was also no effect of *M. bovis* on the likelihood of being a *H. bakeri* super-shedder (MB-: 18.5%, MB+: 33%; $X^2_1 = 0.87$, $p = 0.35$).

Helminth-M. bovis co-infection: Effects on Host

M. bovis co-infection effects were strongly manifest in the host, but these effects depended on diet. Overall, MB- mice gained more weight than MB+ mice ($F_{1,115} = 13.5$, $p < 0.001$), but helminth infection interacted with *M. bovis* infection such that only COINF MB- mice gained more weight than COINF MB+ mice ($F_{3,115} = 5.37$, $p < 0.01$; Fig. 4.3C).

M. bovis infection affected both lung and intestinal immunity, and these effects once again depended on diet. Histopathological eosinophil scores did not differ between MB- and MB+ mice in either the intestine or lung (Table 4.1), but differences were detected by flow cytometry (Table 4.2). Specifically, among mice fed the low diet, MB+ mice had lower levels of intestinal eosinophils than MB- mice ($p < 0.025$; Fig. S4.3). *M. bovis* infection interacted with helminth infection, such that among MB+ mice, intestinal eosinophils were higher in HB mice than CTL mice ($p < 0.01$), and in COINF mice compared to NB mice ($p < 0.025$) and CTL mice ($p < 0.001$). Among MB- mice, no differences were detected between helminth treatments. Lung eosinophil levels measured using flow cytometry were higher in MB+ mice compared to MB- mice (Table 4.2; Fig. S4.3).

Structural equation models

Structural equation models evaluated how treatments (co-infection and diet) influenced immunity, resources, and helminth egg shedding. The lack of significant differences between the final models and the egg shedding data indicate a good model fit (*Nb*: $X^2 = 7.56$, df = 7, $p = 0.37$; *Hb*: $X^2 = 7.49$, df = 7, $p = 0.38$). The effects of diet on egg shedding were likely manifest via host immunity rather than host resources (Fig. 4.1B,C). Both *N. brasiliensis* and *H. bakeri* egg shedding were negatively affected by immunity, which in both models was negatively affected by diet and positively affected by *M. bovis* co-infection, suggesting pathways for indirect immune-mediated interactions (Fig 4.1B,C). Unlike the traditional analyses, the SEM also detected a direct, positive link between *H. bakeri* co-infection and *N. brasiliensis* egg shedding (Fig 4.1B). No significant resource-mediated direct or indirect effects on egg shedding or immunity were detected (Fig 4.1B,C).

Discussion

The goals of this study were to examine how host resource limitation influences the outcomes of co-infection, and to explore potential underlying mechanisms for these outcomes. Using factorial combinations of two dietary protein levels and three dissimilar parasites, we found that resources influenced indirect, immune-mediated interactions among co-infecting parasites via two different mechanisms. In contrast, we found no evidence of direct, resource-mediated interactions. During resource limitation, immune-mediated facilitation between the two helminths had a weaker effect on helminth reproductive output, but lengthened infection duration. Conversely, during resource limitation, immune-mediated facilitation between a helminth (*N. brasiliensis)* and microparasite (*M. bovis*) was apparent, increasing helminth reproduction and lengthening infection duration. Using SEMs, we found support for the hypothesis that resources affected immunity, and that immunity was the stronger driver of variability in parasite fitness. There was no support for direct, resource-mediated interactions among parasites because egg shedding of neither helminth species was influenced by resources. For hosts, parasite infection affected performance, but resource limitation only influenced weight gain from days 0 to 6. Hosts gained less weight during periods of peak helminth egg shedding and hosts infected with all three parasites gained the least weight.

Helminth co-infection

The effects of helminth co-infection were strongly asymmetrical, with higher *Nippostrongylus brasiliensis* fitness (persistence and fecundity) in co-infected hosts, but no effects of co-infection on *H. bakeri*. Co-infected hosts took an average of five days longer to clear *N. brasiliensis* infections and shed 7-fold more eggs. Conversely, *Heligmosomoides p.*

bakeri egg shedding did not differ between COINF and HB mice. Furthermore, the likelihood of being a *N. brasiliensis* super-shedder was higher in COINF mice compared to NB mice, but variability in *H. bakeri* egg shedding did not differ between HB and COINF mice. Our data add to the many laboratory and field studies documenting positive, immune-mediated effects of *H. bakeri* co-infection on Th2-stimulating macroparasites including *N. brasiliensis* (Colwell and Wescott 1973, Behnke et al. 2009, Ferrari et al. 2009, Maizels et al. 2012). However, while co-infection has been identified as a factor that can potentially generate super-shedders (Stein 2011), only a handful of studies have documented such effects (Sherertz et al. 1996, Cattadori et al. 2008, Lass et al. 2013). Our results lend support to this idea, and suggest that higher *N. brasiliensis* egg shedding rates among co-infected individuals could increase population-level parasite transmission by affecting both the number of infective stages in the environment and the variance in egg shedding among host individuals.

The pattern of higher and longer duration *N. brasiliensis* egg shedding in COINF mice is consistent with indirect, immune-mediated, facilitative interactions between *H. bakeri* and *N. brasiliensis*, a mechanism that is supported by immunological data. *H. bakeri* is known to stimulate the cytokine IL-10 and recruit Treg cells, which are immunosuppressive and dampen Th2 responses (Maizels et al. 2012). In the lung, where *H. bakeri* was absent, both NB and COINF mice had higher histopathological eosinophil scores than HB and CTL mice. However, in the intestine where *H. bakeri* was present, COINF had lower intestine eosinophil scores than NB mice. This difference between COINF and NB mice was present at D14 during peak *N. brasiliensis* egg shedding, but not at D28 after *N. brasiliensis* clearance. Thus, the immune-mediated facilitation was limited to the specific time period and site of co-infection. Although we did not measure T-cell subsets directly, since eosinophil infiltration is triggered by cytokines

produced by Th2 cells (Janeway 2008), the observed pattern of lower eosinophil recruitment in COINF mice supports the idea that the mechanism underlying the facilitative interaction between *H. bakeri* and *N. brasiliensis* was an *H. bakeri*-driven reduction of the Th2 immune response. While traditional analysis supported indirect immune-mediated interactions between the helminths, the SEM revealed a direct, positive link between H. bakeri co-infection and *N. brasiliensis* egg shedding, and no evidence for indirect effects mediated by immunity or resources. However, the SEM analysis only included immunological data from D28, rather than D14, when patterns of immune-mediated facilitation were detected using traditional analysis.

Although co-infection with *H. bakeri* facilitated *N. brasiliensis* infection among mice fed both diets, we found evidence of weaker facilitative interactions in hosts fed low protein diets compared to standard protein diets. *N. brasiliensis* infection duration was longer in co-infected hosts fed the low diet compared to the standard protein diet, but *N. brasiliensis* reproduction showed the opposite pattern. Specifically, co-infection with *H. bakeri* resulted in 6.4-fold lower *N. brasiliensis* egg shedding in hosts fed a low diet than hosts fed a standard protein diet. Interestingly, the SEM analysis did not detect the context-dependent interaction between diet and co-infection because it averaged across all treatment combinations. Consistent with the observed effect of resources on helminth egg shedding, COINF and NB mice on a low diet had nearly identical levels of eosinophils, whereas COINF mice on a standard protein diet had lower eosinophil levels than NB mice on the same diet. Although this immunological effect was not significant ($p = 0.20$), these data support the prediction that resource limitation dampened the immune-mediated facilitation apparent among COINF mice fed the standard protein diet.

M. bovis co-infection

Our data on helminth-*M. bovis* co-infection suggest that protein malnutrition strongly affects the outcome of interactions between helminths and microparasites. *M. bovis* infection had a positive effect on *N. brasiliensis*, but only when host resources were limited. Among mice fed the low diet, *M. bovis* co-infection led to several fold increases in *N. brasiliensis* egg shedding and prolonged infection durations in both NB and COINF mice (9.3 and 3.5-fold, respectively). The resource-dependency of microparasite-macroparasite interactions has not previously been demonstrated experimentally, although a supporting pattern has been observed in a cross-sectional study of African buffalo where a tradeoff in Th1and Th2 immune defenses was detectable only during the dry season when resources are extremely limited (Jolles et al. 2008, Ezenwa and Jolles 2011). In human populations around the world, *M. tuberculosis* infection has consistently been associated with lower levels of serum albumin, an indicator of protein status. Likewise, better nutrition is often associated with faster tuberculosis recovery (van Lettow et al. 2003). The *N. brasiliensis* response to *M. bovis* co-infection suggests that co-infection outcomes may change when hosts are forced to allocate differing resource pools to competing physiological demands. In contrast, there was no effect of *M. bovis* on *H. bakeri*. In direct contrast to our *H. bakeri* result, Lass et al. (2013) found that mice co-infected with respiratory bacterial pathogen were more likely to be *Heligmosomoides polygyrus* super-shedders than singly-infected mice. *H. bakeri* induces a relatively weak Th2 response during primary infection (Maizels et al. 2012), so the lack of top-down Th1-Th2 interactions between a Th1-inducing microparasite and *H. bakeri* is not surprising, although not unprecedented (Lass et al. 2013). Our experimental results demonstrate that effects of *M. bovis* on helminth egg shedding are possible,

but not universal consequences of helminth-microparasite co-infection, and that the resources available to hosts may strongly influence co-infection outcomes.

The dependence of the helminth-*M. bovis* interaction on diet was corroborated by immunological data. Immune responses detected using flow cytometry were consistent with the observed patterns of helminth egg shedding and were more sensitive to the effects of diet and *M. bovis* co-infection than histopathology data. For mice fed the low diet, lung eosinophil levels were lower in MB+ mice compared to MB- mice, suggesting a trade-off between the Th1 response to *M. bovis* infection and the Th2 response to helminth infection under low resource conditions. Furthermore, the Th1-Th2 trade-off was not localized to the site of *M. bovis* infection; MB+ mice also had lower intestinal eosinophil levels than MB- mice. The lower eosinophil responses following *M. bovis* co-infection are consistent with the higher *N. brasiliensis* egg shedding and infection duration noted in mice fed the low diet. Interestingly, mice fed the standard protein diet had higher lung and intestinal eosinophil levels when co-infected with *M. bovis*, yet these differences did not translate into lower helminth egg shedding or shortened infection duration compared to MB- mice. Together, these data support the potential systemic, rather than site-specific, nature of some co-infection-induced immune interactions.

Both the *N. brasiliensis* and *H. bakeri* SEMs suggest causal pathways by which *M. bovis* co-infection could indirectly influence helminth egg shedding. *M. bovis* co-infection was associated with higher lung eosinophil levels and higher immune function. Both *N. brasiliensis* and *H. bakeri* egg shedding were negatively influenced by immunity in the SEMs. Consequently, via immunity, *M. bovis* co-infection could indirectly lead to lower egg shedding for both helminth species. However, the traditional analyses showed that *M. bovis* co-infection was associated with higher *N. brasiliensis* egg shedding during resource limitation. These

contradictory results may indicate a weakness of the SEM framework, which could not incorporate the context-dependent, immune-mediated interaction between *M. bovis* and *N. brasiliensis* during resource limitation detected using traditional analysis. Despite these differences, both the SEM and traditional analyses point to strong immune-mediated interactions between *M. bovis* and helminths that have the potential to influence helminth transmission.

Effects on hosts

Diet and co-infection had strong effects on the parasites, but more variable effects on the host. Mice fed the standard protein diet gained a higher percentage of their initial body weight compared to mice fed the low diet during the first week of the experiment, but diet had no effect on subsequent weight gain. The two diets had nearly identical caloric content (SP: 4.09 kcal/g, LP: 4.11 kcal/g gross energy), so it is not surprising that weight gain over the course of the experiment was similar. Conversely, parasite infection had strong effects on weight gain. Mice gained less weight during the periods of peak egg shedding for each helminth species. *M. bovis* infection led to similar-magnitude decreases in weight gain. While the consequences of single infections for the host were clear, parasite co-infections showed little effect. The notable exception was that co-infection with all three parasites resulted in a 3-fold reduction in weight gain compared to infection with the two helminths. Since organisms have a limited amount of energy available to invest in growth versus immunity and reproduction (French et al. 2009), weight gain may reflect an integrated cost of infection that includes energy lost directly to the parasite, tissue repair, altered nutrient absorption efficiency, or immune responses. In wild mice, female weight is strongly correlated with lifetime reproductive success, so weight gain is likely a meaningful indicator of the fitness consequences of infection (Ribble 1992). As such, our results

strongly suggest that certain combinations of co-infections may have significant fitness consequences for the host that go beyond the negative effects of single infection.

An intriguing finding with respect to host immunity was that low mice had stronger immune responses and lower parasite burdens than standard protein mice. Protein supplementation is often associated with higher levels of eosinophils and other immune mediators (Coop and Kyriazakis 2001, Koski and Scott 2001). Similarly, one might expect animals with higher protein diets to have lower parasite burdens, but instead, we observed higher eosinophils and lower *N. brasiliensis* egg shedding during protein limitation. The opposing host responses to protein supplementation versus limitation suggest that the relationship between protein and immune responsiveness is non-linear. The observed difference in immune function and egg shedding during standard and protein-limited conditions may reflect outcomes of tolerance and resistance strategies, respectively. The higher eosinophil levels and lower egg shedding levels among mice fed the low diet fit predictions of a resistance strategy, where hosts expend energy to control infections. By contrast, the lower eosinophil levels and higher egg shedding among mice fed the standard protein diet fit predictions of a tolerance strategy, where hosts incur the energetic costs of infection rather than expend resources to control the infection. In support, it is well known that protein supplementation enables sheep and goats to tolerate helminth infections without declines in weight or milk production (Coop and Kyriazakis 2001). Similarly, standard protein mice showed no decline in weight gain associated with increased egg shedding, suggesting tolerance. By contrast, weight gain declined precipitously with increasing egg shedding in low mice. However, on average, low and standard protein mice had equivalent weight gains throughout the experiment, suggesting that these strategies were energetically equivalent.

The effects of host nutrition on immunity and infection are clearly complex and require comprehensive investigation that includes consideration of parasite tolerance as a response to varying resource levels. Such additional studies are warranted because the ecological and management implications of an interaction between resource availability and/or nutrition and resistance/tolerance strategy are sizable. If hosts adopt a tolerance strategy under low resource conditions or are simply unable to mount an effective immune response, resource supplementation could increase individual resistance and reduce parasite transmission. However, if hosts adopt a tolerance strategy under high resource conditions, as observed in this study, resource supplementation may unwittingly increase parasite transmission if hosts relax immunological control of the parasite. By influencing the propagation of parasites within hosts, the relationship between nutrient limitation and parasite defense strategy could have serious implications for disease transmission in humans, domestic animals, and wildlife.

Conclusions

While separate immune- and resource-mediated effects on co-infecting parasites have been detected in previous studies (Graham 2008), this is the first experiment to explicitly test their relative importance for parasite fitness. SEM analysis allowed us to determine that indirect, immune-mediated interactions had the strongest effects on helminth fecundity in our study. Interestingly, a recent meta-analysis found that resource-mediated interactions are most common in human co-infections, but the strength of different interactions could not be accounted for due to a lack of requisite data (Griffiths et al. 2014). We also found that parasite fitness and host performance strongly depended on diet quality and the combination of co-infecting parasites. Immune-mediated facilitation between micro- and macroparasite infection was stronger during

resource limitation. Resource scarcity and helminth infection also frequently co-occur in human populations, and our data suggest those populations may also be more vulnerable to microparasitic infection. Overall, our findings highlight that the outcomes of co-infection are context-dependent for both parasites and hosts, and that resources are a key context that shapes the magnitude and direction of parasite interactions.

Acknowledgments

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Tables

Table 4.1. Effects of resources, helminth infection (NB, HB, COINF, CTL), and microparasite infection (MB+, MB-) on histopathology on days 14 (D14) and 28 (D28).

	df	D14 Intestine	D14 Lung	D28 Intestine	D28 Lung
Protein	1	6.31*	0.12	16.5***	0.15
Helminths	3	9.16*	48.7***	20.3***	68.2***
Microparasite	1			0.06	0.61

Data are X^2_{df} values with their significance denoted with asterisks: $p < 0.05$*, <0.01**, <0.001***

Table 4.2. Effects of resources and co-infection on the relative abundance of eosinophils measured using flow cytometry.

	df_1	D14 Intestine	D14 Lung	D28 Intestine	D28 Lung
Protein	1	4.06*	6.56*	30.9***	25.7***
Helminths	3	26.6***	6.73***	10.8***	4.26**
Protein * Helminths	3		8.49***	3.25*	1.66
Microparasite	1			0.77	44.6***
Protein * Microparasite	1			12.8***	0.01
Helminths * Microparasite	3			3.07*	0.57

Data are X^2_{df1} (D14 intestine) or $F_{df1,df2}$ (D14 lung: $df_2 = 56$, D28: $df_2 = 112$) values.
Significant values are denoted with asterisks: $p < 0.05$*, <0.01**, <0.001***.

Figure Legends

Figure 4.1. The initial model (A) for *Nippostrongylus brasiliensis* (*Nb*) and *Heligmosomoides p. bakeri* (*Hb*) differed only by which was the focal species for egg shedding (1) and which was the co-infecting parasite (2). In the final models for B) *N. brasiliensis* and C) *H. bakeri* egg shedding, standardized path coefficients are noted beside connections, and asterisks indicate the level of significance. Non-significant effects are indicated by dashed lines. Black lines indicate positive effects and gray lines indicate negative effects. The model's explanatory power (R^2) is noted above the response variables.

Figure 4.2. Helminth egg shedding (± 1 se) of mice infected with *N. brasiliensis* (NB), *H. bakeri* (HB), or both helminths (COINF) and fed either a standard protein (SP) diet or a low protein (LP) diet. Mice were infected with helminths on D7 and *Mycobacterium bovis* on D14 (grey arrows). The inset graphs show total egg production (± 1 se) for each helminth species by diet treatment.

Figure 4.3. Mean weight gain (± 1 se) for mice culled on A) D6, B) D14, and C) D28. For B) and C), differences among helminth treatments are shown with uppercase letters, while differences within treatments are shown by lowercase letters. If no letters are shown, there are no differences among treatments.

Figure 4.4. The relationship between A) total *N. brasiliensis* egg shedding and B) total *H. bakeri* egg shedding and total host weight gain (g) differed between the low protein (LP) and standard protein (SP) diets.

Figure 4.5. *N. brasiliensis* (*Nb*) egg shedding (± 1 se) was higher in *M. bovis* coinfected hosts (MB+) only when hosts were fed a low protein (LP) rather than a standard protein (SP) diet.

Figure S4.1. Egg size distributions for *N. brasiliensis* (n = 32 eggs from 4 mice) and *H. bakeri* (n = 199 eggs from 6 mice).

Figure S4.2. Mean histopathological eosinophil scores (± 1 se) by helminth treatment on D14 and D28. Letters denote significant differences among helminth treatments.

Figure S4.3. Eosinophil percentages in the intestinal mucosa and lung measured using flow cytometery.

Figures

Figure 4.1

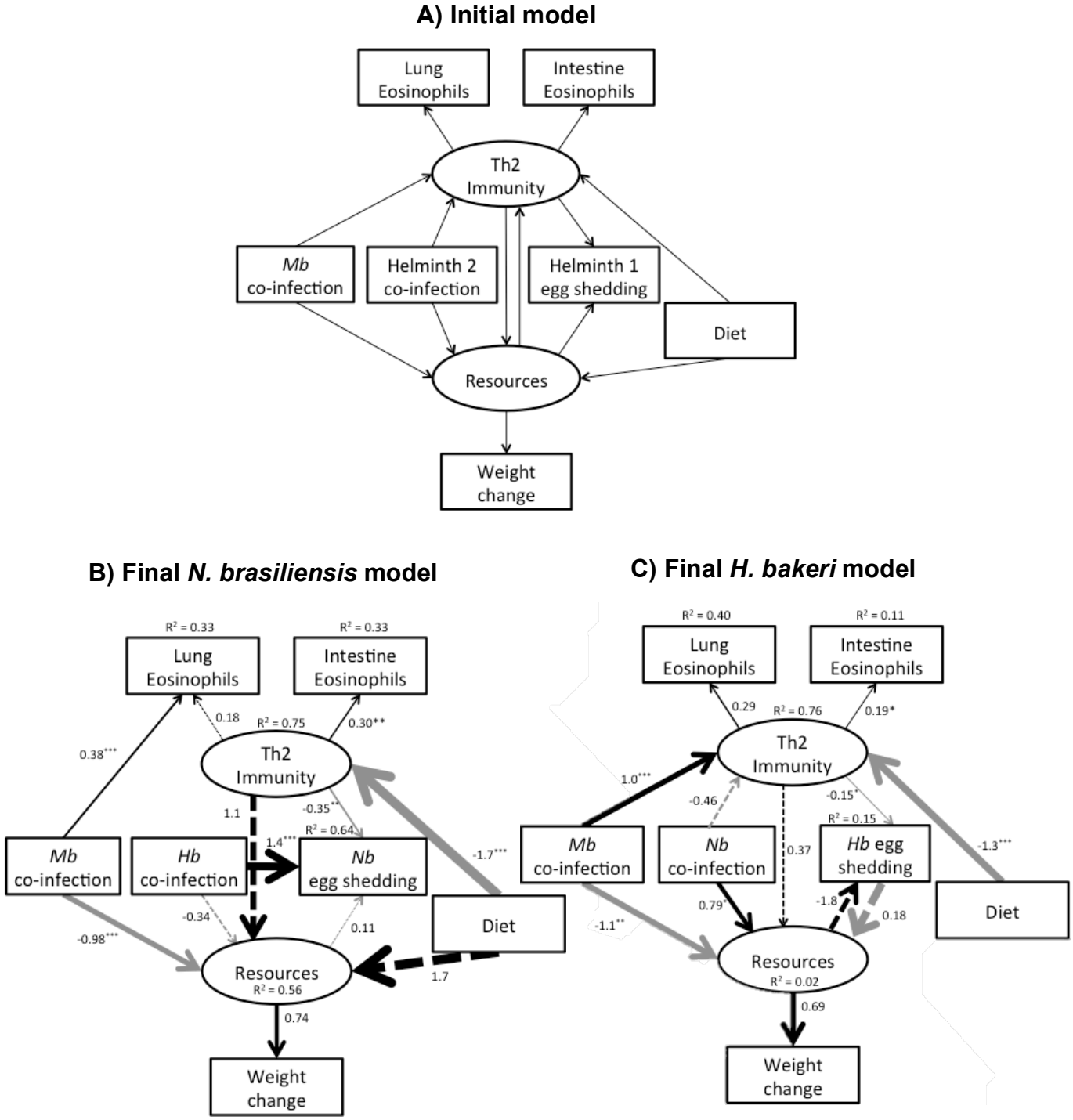


Figure 4.2

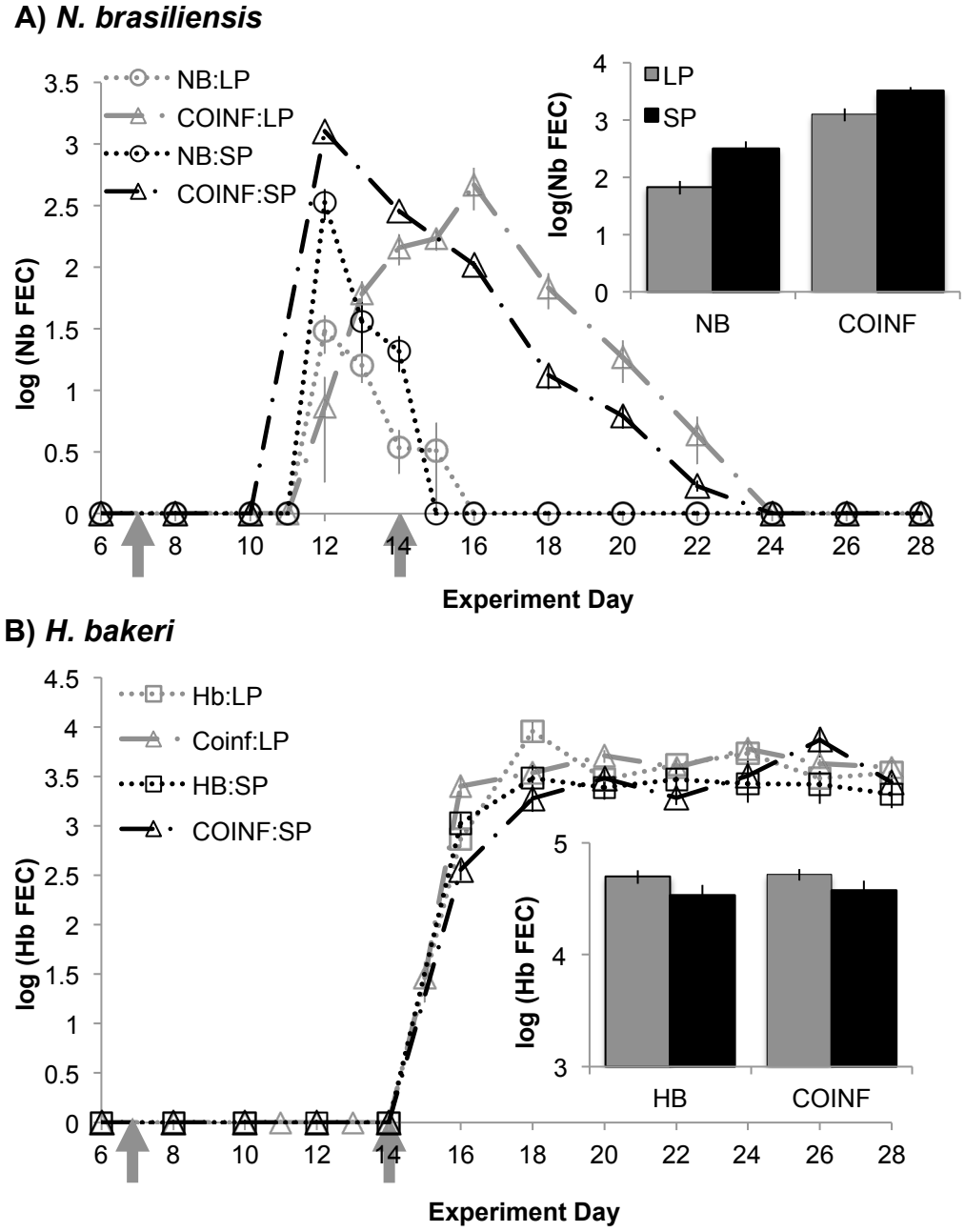

A) N. brasiliensis
NB:LP
COINF:LP
NB:SP
COINF:SP
log (Nb FEC)
Experiment Day
LP
SP
log(Nb FEC)
NB
COINF
B) H. bakeri
Hb:LP
Coinf:LP
HB:SP
COINF:SP
log (Hb FEC)
Experiment Day
log (Hb FEC)
HB
COINF

Figure 4.3

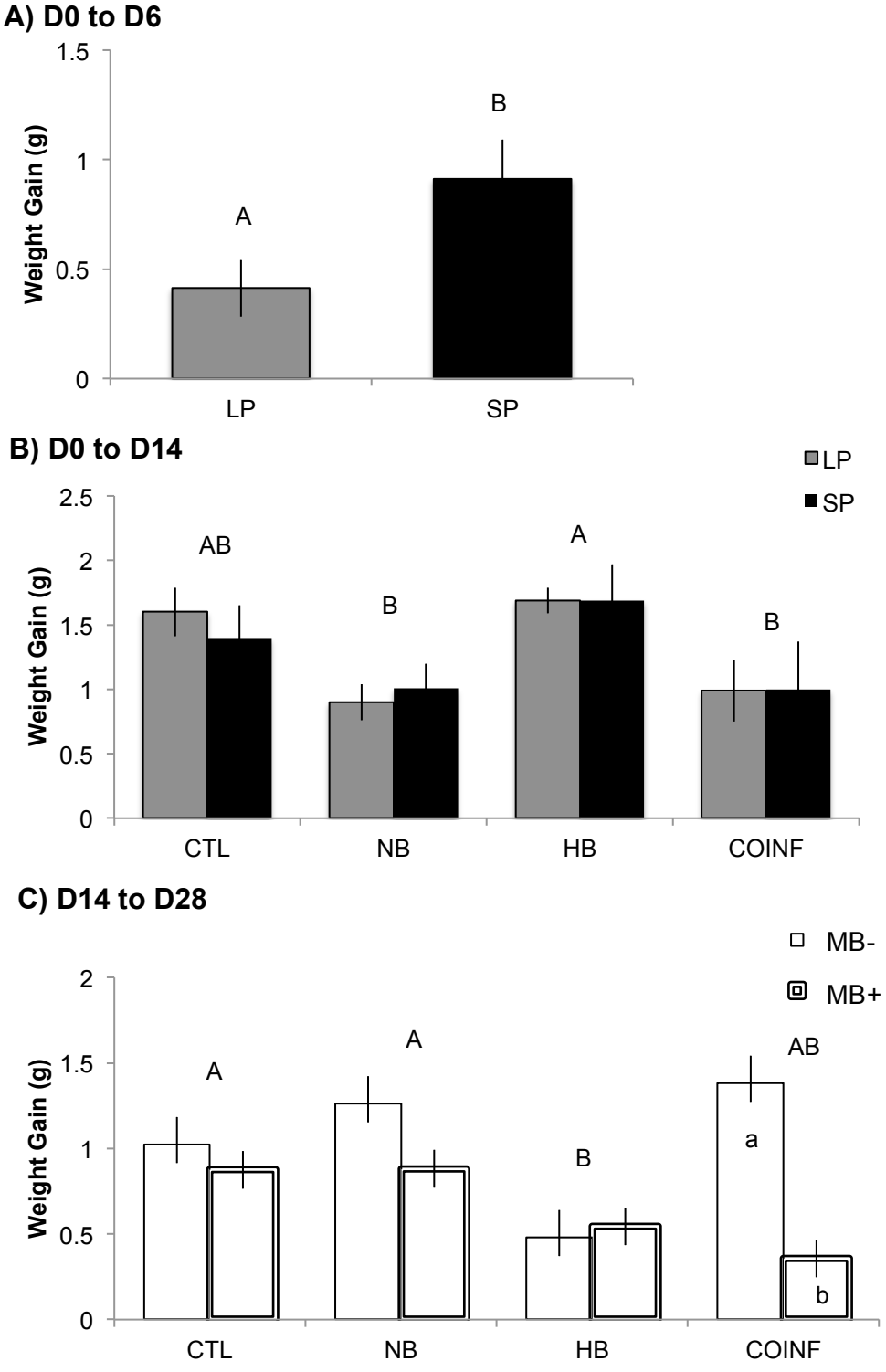
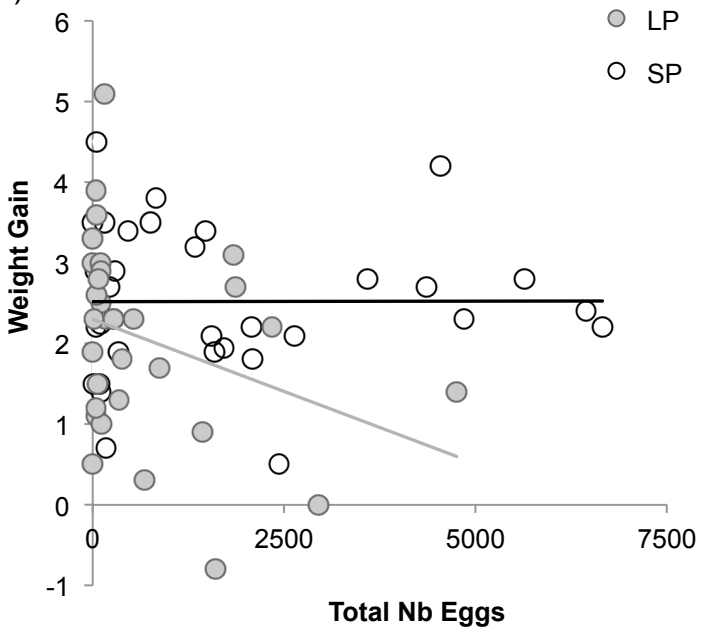

A) D0 to D6
Weight Gain (g)
1.5
1
0.5
0
A
B
LP
SP
B) D0 to D14
LP
SP
Weight Gain (g)
2.5
2
1.5
1
0.5
0
AB
B
A
B
CTL
NB
HB
COINF
C) D14 to D28
MB-
MB+
Weight Gain (g)
2
1.5
1
0.5
0
A
A
B
AB
a
b
CTL
NB
HB
COINF

Figure 4.4

A) *N. brasiliensis*-infected



B) *H. bakeri*-infected

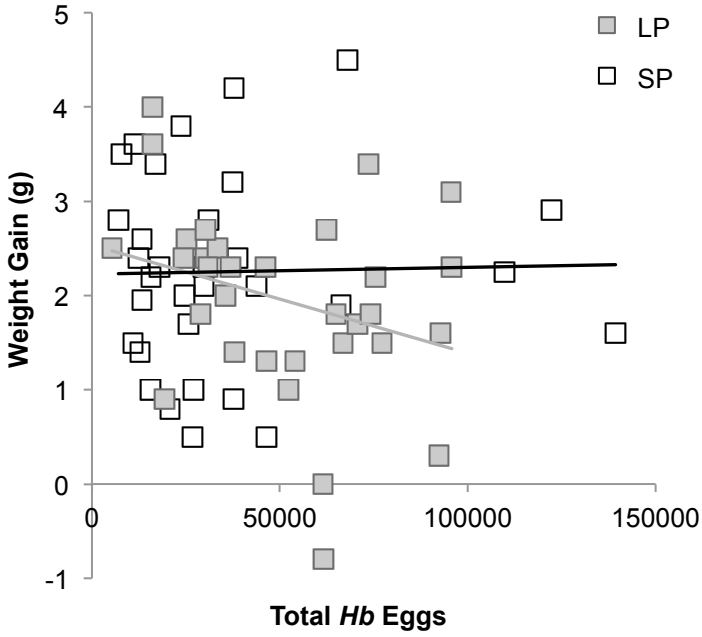


Figure 4.5

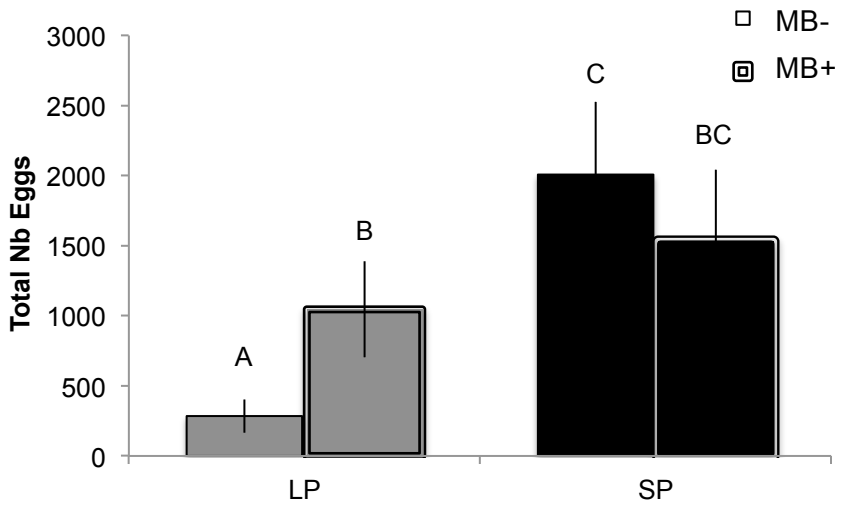


Table S4.1. In the lungs, the number of eosinophils cuffing medium-caliber blood vessels was scored on a scale of 0 to 4. Intestinal slides were also scored on a scale of 0 to 4 for the number of eosinophils per villus infiltrating the lamina propria.

Score	**0**	**1**	**2**	**3**	**4**
Lung	none	1-5	6-10	11-15	> 15
Intestine	none	1-2	3-5	6-10	> 10

Figure S4.1

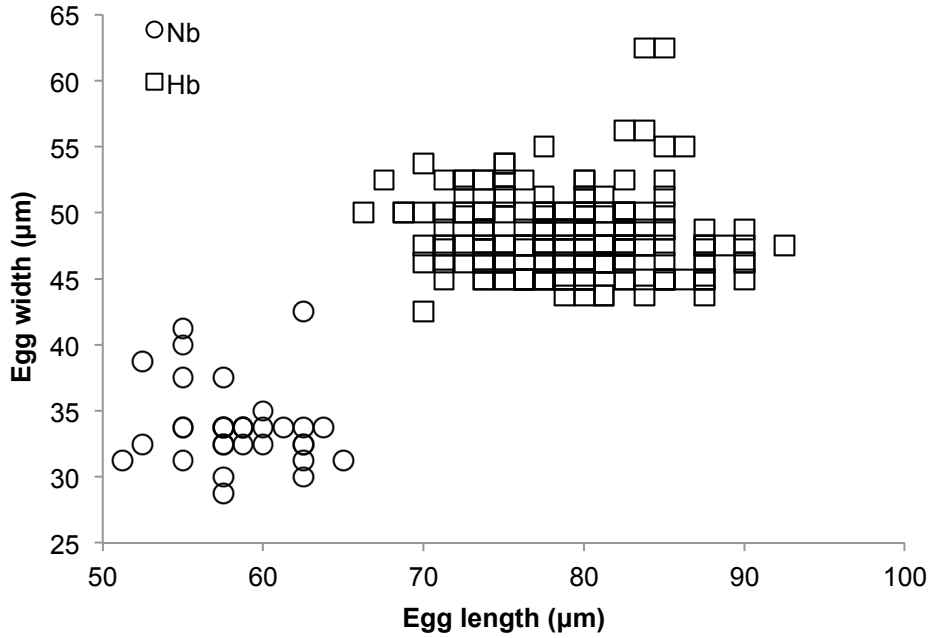

Nb
Hb
Egg width (µm)
Egg length (µm)
65
60
55
50
45
40
35
30
25
50
60
70
80
90
100

Figure S4.2

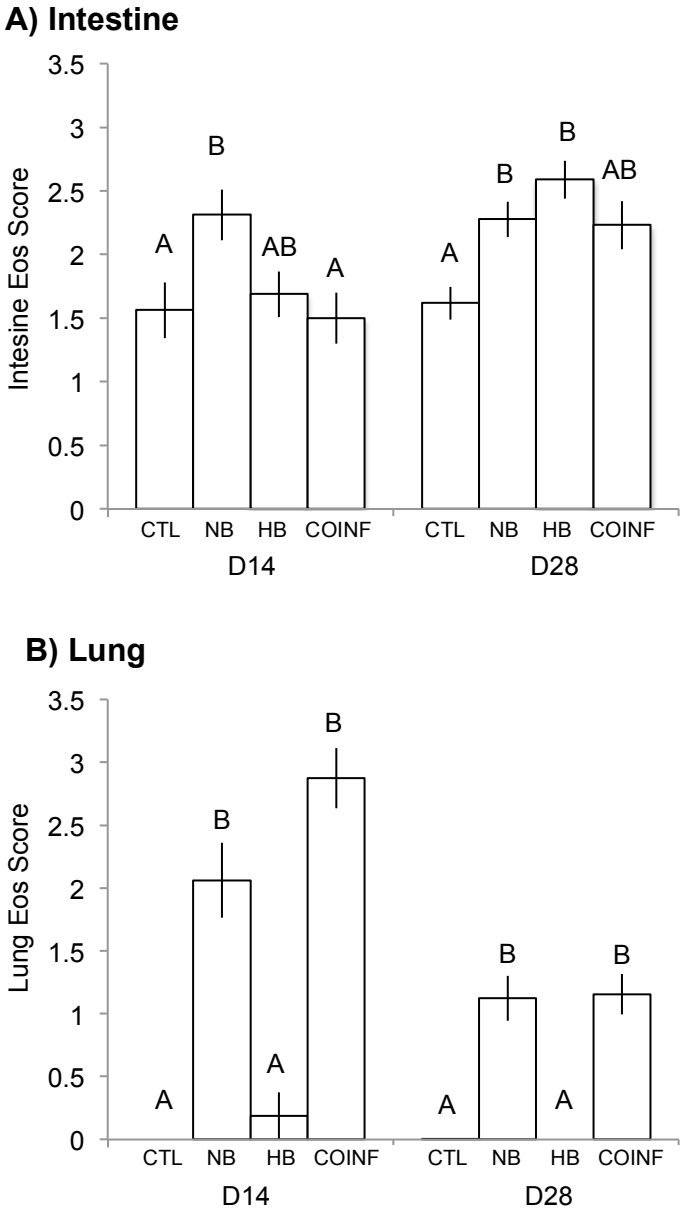
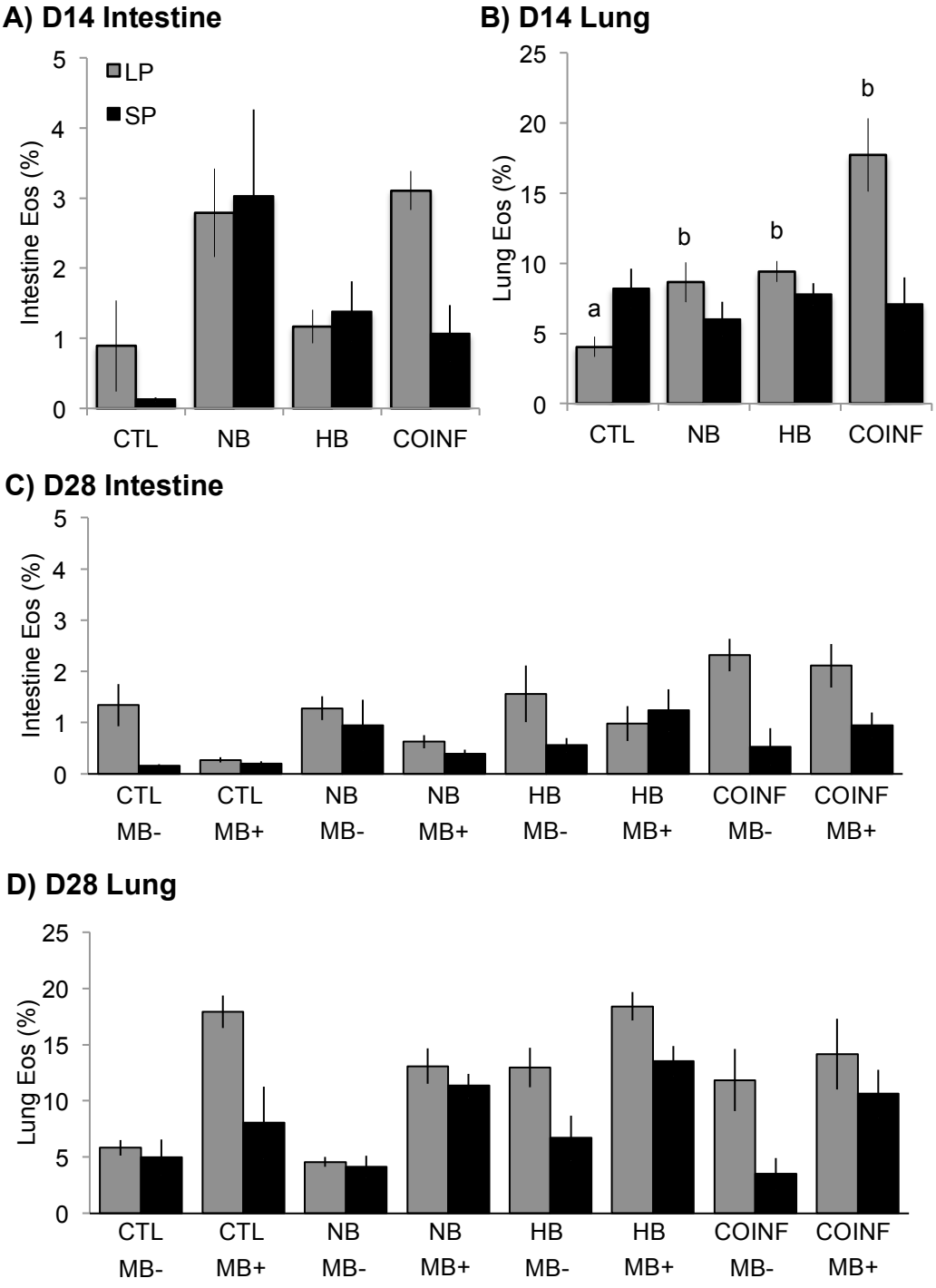

A) Intestine
Intesine Eos Score
3.5
3
2.5
2
1.5
1
0.5
0
A
B
AB
A
A
B
B
AB
CTL NB HB COINF
CTL NB HB COINF
D14
D28
B) Lung
Lung Eos Score
3.5
3
2.5
2
1.5
1
0.5
0
A
B
A
B
A
B
B
CTL NB HB COINF
CTL NB HB COINF
D14
D28

Figure S4.3


A) D14 Intestine
LP
SP
Intestine Eos (%)
0
1
2
3
4
5
CTL
NB
HB
COINF
B) D14 Lung
Lung Eos (%)
0
5
10
15
20
25
a
b
b
b
CTL
NB
HB
COINF
C) D28 Intestine
Intestine Eos (%)
0
1
2
3
4
5
CTL MB-
CTL MB+
NB MB-
NB MB+
HB MB-
HB MB+
COINF MB-
COINF MB+
D) D28 Lung
Lung Eos (%)
0
5
10
15
20
25
CTL MB-
CTL MB+
NB MB-
NB MB+
HB MB-
HB MB+
COINF MB-
COINF MB+

CHAPTER 5

A TALE OF TWO WORMS: PARASITE SPECIES IDENTITY DETERMINES FITNESS COSTS OF INFECTION[1]

[1] Budischak, S. A., A. E. Jolles, and V. O. Ezenwa

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Abstract

The fitness costs of parasite infection are poorly described for wild hosts where coinfection with multiple sub-lethal parasites is common. Using a novel sub-lethal sampling approach, we tracked helminth species abundances through time in African buffalo. We examined patterns of association between the abundance of each helminth species and host condition, immunity, microparasite infection, reproduction, and survival. While the changes in the abundance of one helminth species (*Haemonchus sp.*[H]) were associated with declines in host body condition and increasing immune cross-regulation, changes in the other helminth (*Cooperia fulleborni* [Cf]) were associated with increases in host condition and earlier onset of reproduction. Importantly, hosts in poor condition were less likely to survive, and because Cf and H had opposing consequences for host condition, they may also have contrasting indirect effects on host survival. Neither helminth had direct effects on coccidia infection, but H may have indirect effects on coccidia via host immunity. Specifically, H infection skewed host innate defenses toward Th2 responses, and aspects of Th2 immunity were positively associated with coccidia abundance. This work reveals the potentially cryptic and complex effects of multiple helminth infections on host fitness.

Introduction

Parasites, by definition, exploit their hosts, but the fitness costs of parasite coinfection are largely unknown for wild hosts where mixed infections with multiple sub-lethal parasites is the norm (Bordes and Morand 2011). Understanding the effects of parasite community composition on host fitness is of both applied and theoretical importance because environmental changes and new species introductions are impacting both parasite and host communities, generating novel

host-parasite combinations (Lafferty et al. 2008, Telfer and Bown 2012). The consequences of coinfection can be dramatic; colony collapse disorder, the emergence of virulent coral pathogens, lion die-offs due to canine distemper virus, and the global rise in tuberculosis have all been attributed to multi-parasite infections (Corbett et al. 2003, Munson et al. 2008, Bromenshenk et al. 2010, Godwin et al. 2012). Outside of laboratory experiments, the fitness costs of infection are difficult to quantify because hosts in poor condition are often infected with multiple parasites and it is difficult to ascertain which parasite (or combination of parasites) impacted host condition, or if poor condition hosts were simply more susceptible to infection (Beldomenico and Begon 2010).

Synergistic and antagonistic interactions among co-occurring parasites can affect infection duration, infectiousness, and disease severity (Lello et al. 2004, Balmer et al. 2009, Telfer et al. 2010). One potential repercussion of infection by some parasite species is increased susceptibility to other parasites (Viney and Graham 2013). HIV/AIDS is perhaps the most dramatic example of the importance of parasite-induced changes in susceptibility for host survival (Lane et al. 1994), but many other parasites also have effects on susceptibility to other parasites and pathogens (Ferrari et al. 2009, Ezenwa et al. 2010, Telfer et al. 2010). A small but increasing number of empirical and theoretical studies suggest that the individual-level effects of coinfection significantly impact host and parasite populations (Fenton 2008, Lello et al. 2008, Ezenwa and Jolles 2011). For example, interactions among coinfecting parasites can influence the ability of hosts to resist further infections (Cattadori et al. 2007, Ezenwa et al. 2010) and for parasites to expand their geographic and host ranges (Ezenwa and Jolles 2011, Telfer and Bown 2012).

In particular, helminth coinfection is receiving considerable attention because immune defenses to macroparasites (T-helper type 2 [Th2]) trade-off with microparasite defenses (T-helper type 1 [Th1]). Resultantly, helminth infection can affect the morbidity, mortality, progression, and transmission of important human and wildlife microparasitic diseases (e.g. HIV, malaria, tuberculosis) (Jolles et al. 2008, Ezenwa et al. 2010, Salgame et al. 2013). Wildlife studies experimentally removing entire helminth communities have documented strong effects of helminths on microparasite infection (Ezenwa et al. 2010, Knowles et al. 2013, Pedersen and Antonovics 2013), condition (Stien et al. 2002, Newey et al. 2005, Ezenwa et al. 2010), survival (Gulland 1992), and reproduction (Stien et al. 2002, Ballesteros et al. 2012). Notably, even closely related helminth species can differentially affect host immune function, microparasite infection risk, and host performance (Budischak et al. 2012)(Budischak in prep - Ch 4). For example, removal of some nematode species decreases HIV replication in humans (Walson and John-Stewart 2007), but other species have no effect on HIV progression (Brown et al. 2004, Modjarrad et al. 2005). This variation may be due to species-specific differences in host immune responses; a meta-analysis of coinfection experiments in laboratory mice found that the magnitude of a helminth's effect on microparasite infection was proportional to its effect on Th1 immunity (Graham 2008). Helminth community composition typically varies among individuals, across populations, and through time, but current treatments are unable to selectively manipulate individual members of the helminth community. Thus, understanding consequences of helminth coinfection in natural populations requires additional research into the effects of intact, but variable, helminth communities on microparasite susceptibility and host fitness.

To explore links between helminth infection and components of host physiology and fitness, we conducted a 4-year longitudinal study of the gastrointestinal (GI) parasites of African

buffalo (*Syncerus caffer*) using a unique molecular approach to distinguish helminth species shed in the feces of live hosts. We examined how changes in the abundances of individual helminth species were associated with changes in host condition, immune function, microparasite infection, reproduction, and survival (Fig. 5.1). Previous cross-sectional work in this host species revealed that the two most common helminth types are blood-feeding GI nematodes in the genus *Haemonchus* [H], and a more abundant GI nematode, *Cooperia fulleborni*. These two parasites were shown to have opposing effects on physiological indicators of host performance and immune function, such that effects of the more pathogenic but less abundant species, *Haemonchus,* were masked when considered in combination with the more abundant species, *Cooperia fulleborni* (Budischak et al. 2012). Because prior work focused on the impacts of parasites using samples from a single time point, here our goal was to investigate how changes in parasite infection through time were associated with changes in host condition, immunity, reproduction over a longer timescale. Our longitudinal approach evaluated whether increases or decreases in the abundance of specific worm species corresponded to simultaneous positive or negative changes in host traits. Although this approach is correlational, it enables detection of species-specific effects that are potentially masked during experimental removal studies and provides stronger causal inference than single time-point studies.

Based on our prior work, we expected that the two worm species would have distinct consequences for host fitness, with H having negative effects on host reproduction or survival, possibly mediated by effects on host condition, and Cf having negligible effects on host fitness, given the previously observed positive associations between Cf and host body condition (Budischak et al. 2012). By taking a species-specific approach to investigating helminth

infection, this work will provide insight into the cryptic and complex effects of multiple helminth infections on host fitness.

Materials and Methods

Field study

African buffalo were sampled in Kruger National Park (KNP), a 19,000 km^2 preserve in northeastern South Africa. The park experiences distinct wet and dry seasons (late wet: Jan-Mar, early dry: Apr-Jun, late dry: Jul-Sept, early wet: Nov-Dec). Initial captures of 100 buffalo each were carried out by South Africa National Parks Veterinary Wildlife Services in June-July 2008 (Lower Sabie herd [LS]) and October 2008 (Crocodile Bridge herd [CB]). The park buffalo herds were located by helicopter and young females (2-5 yr) were preferentially targeted for darting. Animals were recaptured from a vehicle approximately every six months from November 2008 to August 2012. As part of a larger experiment on parasite interactions, half of the buffalo received an anthelmintic treatment at each capture, but these animals were excluded from this study, with the exception of their initial capture, which preceded drug treatment. In total 249 animals were used to examine patterns of infection within a single capture period, and 123 individuals, re-captured between 2-9 times to examine changes in infection, physiology, and fitness over time.

During each capture event, chemically immobilized buffalo were sampled to assess age, reproductive status, and body condition. Age was estimated by incisor eruption and wear for young and old buffalo, respectively (Jolles 2007). Pregnancy status was determined by rectal palpation and lactation was detected via manual milking (Jolles et al. 2005). Condition was assessed using a body condition index (BCI) based on manual palpation and visual assessment of

locations where buffalo store fat (Ezenwa et al. 2009), a technique that has been cross-validated with kidney fat index, a widely-used, but lethal, condition measure (Ezenwa et al. 2009). Scores for each fat deposition site range from 1-5, and mean buffalo condition scores were lowest during the early wet season (mean ± sd; ew: 2.6 ± 0.5, n =152), but mean condition scores were similar in the other seasons (3.1 to 3.3 ± 0.6 to 0.7, n's = 134 to 169). Finally, horn size in African buffalo was negatively correlated with GI parasite infection intensity and lactation status in a previous cross-sectional study (Ezenwa and Jolles 2008), so horn growth measured as the change in horn width between captures was used as an additional, but longer-term, metric of condition.

Immunity and GI parasite infection

To assess potential immune-mediated interactions between helminths and microparasite infection, we measured aspects of Th1 and Th2 immunity in peripheral circulation. Blood samples were collected via jugular venipuncture in EDTA or heparinized tubes and transported to the laboratory on ice. As a measure of innate Th2 defenses, eosinophil counts were performed manually from blood smears by a single observer. Heparinized whole blood was incubated at 37°C for 28 hr, and supernatant plasma was collected and immediately frozen at -20°C for cytokine analysis. Cytokines are signaling molecules that direct the activity of a broad range of immune cells and, separate cytokines coordinate Th1 versus Th2 defenses. Sandwiched enzyme linked immunoabsorbent assays (ELISA) were used to quantify circulating levels of the Th2 cytokine interleukin-4 (IL-4) and the Th1 cytokine interleukin-12 (IL-12), following established protocols (Nemzek et al. 2001). Briefly, 96-well immunoplates were coated overnight with anti-bovine IL-4 or IL-12 polyclonal antibodies (Abd Serotec), and blocked with 5% non-fat dry milk

in PBS, before addition, in duplicate, of plasma samples diluted with phosphate buffered saline (1:40 for IL-4, 1:100 for IL-12). After a 2 hr incubation at room temperature, detection antibodies (mouse anti-bovine IL-4 or IL-12 diluted with PBS to 5 and 2 μg/ml, respectively) were added to each well, and then plates were incubated at 37°C for 1 hr. Concentrations were determined by adding 1:10,000 Strepdavin:HRP in HRP-Stabliplus (Abd Serotec) to each well, stopping the reactions after 10 minutes with 100 μl of 0.2 M sulfuric acid, and reading optical densities at 405 nm with a microplate reader (BioTek). All samples were run in duplicate, and cytokine concentrations (pg/ml) were calculated using IL-4 and IL-12 standard curves generated by performing the above ELISA procedures on serial dilutions of recombinant bovine IL-4 and IL-12. Th1 immunity was further evaluated by the responsiveness of the cytokine interferon-gamma (IFN-γ). Plasma samples are stimulated with a mitogen (pokeweed) and IFN-γ was quantified using a Bovine Interferon gamma ELISA Kit (Abd Serotec). Optical densities were read at 450 nm and compared to a standard curve to determine concentrations (pg/ml).

To assess GI parasite abundance and community composition, a modified McMaster fecal egg counting protocol was used to quantify Strongyle (Nematoda: Trichostrongylidae) nematode eggs and coccidia oocysts (*Eimeria* sp.) (Ezenwa 2003). Coccidia are intracellular protozoan parasites that also inhabit the GI tract and have been documented to respond positively to helminth removal treatment in other mammal species (Knowles et al. 2013, Pedersen and Antonovics 2013). Strongyle fecal egg counts in buffalo are highly correlated with adult worm count (Budischak in prep - Ch. 3), but cannot be used to differentiate among strongyle species. To obtain species-specific strongyle data, we collected larvae from fecal cultures (Archie and Ezenwa 2011, Budischak et al. 2012), extracted DNA (Archie and Ezenwa 2011), amplified the ITS-2 region (Sim et al. 2010), and compared to reference sequences from morphologically

identified adult worms (Budischak in preparation, Ch. 3). Abundance estimates were calculated for the two most common taxa (*Cooperia* fulleborni [Cf] and *Haemonchus sp.* [H]) by multiplying proportional abundance by the total fecal egg count per sample (Budischak et al. 2012). The accuracy of this technique has been verified in buffalo (Budischak in preparation, Ch. 3). The two *Haemonchus* species (*placei* and *bakeri*) were combined for abundance analyses based on previous research suggesting that they have very similar effects on host physiology (Le Jambre 1995). Lastly, we calculated abundance of each species in infected hosts (i.e. infection intensity).

Statistical analysis

First, we used generalized linear mixed models (GLMM) to examine patterns of infection with respect to host age, herd membership, and season. Variation in terms of both the likelihood of infection and abundance within infected hosts, hereafter referred to as intensity, were explored using two-part zero-adjusted hurdle models. Binomial GLMMs with logit links were used to identify the factors influencing infection with Cf, H, or either helminth species. For infected hosts, the effects of season, herd, and age on species-specific infection intensity were tested using truncated negative binomial GLMMs with log links. To account for repeated sampling, animal identity was included as a random effect in all models. Parasite co-occurrence was analyzed following Kuris (Kuris 19990), where observed frequencies of single and coinfections were compared to expected values using a G-test. Pearson correlation tests were used to determine if abundance or change in abundance in Cf and H were correlated.

Next, we tested whether changes in Cf and H abundance influenced changes in host condition and immune function. Separate models were run for each parasite species because Cf

and H abundance were positively correlated (see Results). Changes in each metric were calculated as the estimate at t minus the estimate at t-1. To account for background effects of host and capture traits (e.g. herd, change in season, age, and the interval between captures), all models included these terms in addition to a random effect of host identity. The effects of changes in Cf and H abundance on changes in BCI and horn growth were analyzed using linear mixed models (LMMs) and negative binomial GLMMs (log links), respectively. LMMs were used to test if helminth infection and BCI influenced the change in Th2 immunity measured by eosinophil count, Th1 immunity measured as IFN-γ responsiveness, or the degree of immune cross-regulation measured as the ratio of IL-4 to IL-12. To maintain the directionality of changes in immunity while correcting for leptokurtic distributions, the absolute values for change in eosinophils and IFN-γ were raised to the 0.8 power and changes in IL-4/IL-12 ratios were square root transformed, then each value was multiplied by its original sign. Shapiro-Wilks tests were used to identify the exponential transformation that maximized residual normality.

To examine patterns of coccidia infection, two-part zero-adjusted hurdle models were used to examine prevalence (binomial GLMM with logit link) and intensity in infected hosts (truncated negative binomial GLMM with log link). Coccidia intensity data were square root transformed and two outliers were removed before the truncated GLMM analysis. We used three approaches to determine if helminth abundance, condition, or Th2 immunity influenced infection with coccidia. For the first two approaches, binomial GLMMs with logit links that accounted for capture traits and animal identity were used to test whether changes in Cf or H abundance, BCI, or eosinophils influenced if uninfected hosts became infected and if infected hosts remained infected at the subsequent capture. Thirdly, change in coccidia abundance could not be fit with a GLMM; instead, values were sorted into five groups representing large declines, small declines

(> -250 opg), no change, small gains (< 250 opg), and large gains in coccidia oocyst counts and analyzed using LMMs. Due to sample size constraints, we did not test for effects of IFN-γ or the IL-4/IL-12 ratio on coccidia infection. To account for repeated sampling, animal identity was included as a random effect in all models.

Lastly, we tested whether helminth inflection, condition, or Th2 immunity at one capture influenced survival to or reproductive status at the next capture using binomial GLMMs. Pregnant and/or lactating individuals were classified as reproductive. Non-reproductive young animals were excluded until the time of their first reproduction (17 – 90 mo), with the exception of one outlying individual that did not reproduce until 108 mo. Again, capture traits and a random effect of animal identity were included in all models. We calculated predicted population-level survival odds for the two influential predictors of survival, BCI, and age. Next, we tested whether condition and helminth infection at the prior capture influenced age at first reproduction (measured as age of first pregnancy). Since herd strongly influenced age at first reproduction (CB > LS), separate linear models for the effects of condition and parasite infection were fit for the CB herd vs. the LS and Other [O] herds. All mixed model analyses were run using the lmer (Bates et al. 2004) and ADMB packages in R (Fournier et al. 2012, Skaug et al. 2014).

Results

Patterns of helminth infection

Buffalo were found to be infected with eight species of strongyle nematodes including: *Cooperia fuelleborni* (Cf), *Haemonchus placei*, *Haemonchus bedfordi*, an undescribed species of *Trichostrongylus*, and four other *Cooperia*-like species (n = 778 captures of 249 unique hosts).

Cf was the most prevalent parasite, infecting 38% of hosts at least once during the study. Moreover, Cf infection was detected in 95% of communities sampled from infected hosts. Next most common were the two *Haemonchus* species, which infected a total of 33% of hosts and were present in 65% of helminth communities sampled. T and other *Cooperia*-like species were rarely detected (2 and 3% of hosts, respectively). Hosts were more likely to be coinfected with both Cf and H than expected (Obs. freq: 24%, expected freq: 9.7%, $\chi^2 = 362$, df = 1, $p < 0.0001$). Among all hosts, Cf and H abundance were positively correlated ($r = 0.28$, $t_{776} = 8.0$, $p < 0.0001$), as was change in abundance ($r = 0.17$, $t_{432} = 3.6$, $p = 0.0004$). However, within the subset of infected hosts, neither abundance nor change in abundance were correlated (abund: $r = -0.003$, $t_{311} = -0.04$, $p = 0.97$; Δ abund: $r = 0.13$, $t_{159} = 1.6$, $p = 0.10$).

The likelihood of being infected with Cf and H, and the intensity of H infection decreased with host age (Table 5.1). Infection patterns consistently differed among herds, with LS individuals less likely to be infected with Cf and less intensely infected with H than individuals from the CB and O herds. Additionally, LS and O individuals were more likely to be infected with H than those from the CB herd. Season influenced the likelihood of being infected, but not infection intensity (Table 5.1). Specifically, buffalo were more likely to be infected with Cf in the early wet than the early dry season. H infection was also more common in the early wet season compared to the early dry, as well as the late wet season, and during the late dry compared to the early dry season (Table 5.1). Based on these patterns, we included age, herd, and the change in season between time-points as key covariates in all models of helminth effects on physiological and fitness traits.

Helminth effects on condition

The two nematodes, Cf and H, had opposing associations with host condition. Changes in H abundance were negatively correlated with BCI, suggesting that hosts that gained H lost condition (n = 434 captures of 123 unique hosts; est = -0.0036, t_{425} = -2.9, p = 0.0004). The magnitude of this effect was such that the BCI of a host that increased H intensity by 280 H eggs per gram, would drop a full point on the BCI scale between captures. By contrast, changes in Cf abundance were positively associated with changes in BCI, indicating hosts that gained Cf also gained body condition (est = 0.00039, t_{425} = 2.2, p = 0.029). Although this effect was nearly 10-fold smaller per parasite egg than the negative effect of H, hosts were more heavily infected with Cf. Thus, hosts becoming infected with high numbers of Cf gained nearly half a point on the BCI scale. Change in BCI was also influenced by seasonal changes and capture interval (season: $F_{3,426}$ = 14.1, p < 0.0001interval: est = 0.004, t_{425} = 5.4, p < 0.0001), but not herd or age (p > 0.19). Horn size is an indicator of individual condition and can influence reproductive success in African buffalo (Ezenwa and Jolles 2008). Thus, horn growth gauges the resources an individual has available to invest in this long-term condition indicator. Change in Cf and H abundance were not correlated with horn growth (Cf: est = -0.00037, Z_{425} = -1.1, p = 0.27; H: Est = -0.0014, Z_{425} = -0.90, p = 0.37). Horn growth was influenced by age and capture interval (age: est = -0.03, Z_{425} = -10, p < 0.0001; interval: est = 0.006, Z_{425} = 6.1, p < 0.0001), but not herd or seasonal change (p > 0.10).

Helminth effects on immunity

Changes in H abundance were significantly associated with changes in host immunity, while changes in Cf abundance were not. Increases in H abundance between time points were

associated with declines in eosinophil counts, a measure of innate, Th2 defenses. Additionally, increases in H abundance were associated with increases in the degree of cross-regulation measured as the IL-4/IL-12 ratio (Table 5.2). These links between H abundance and host immunity were robust to the inclusion of changes in condition in the models. Interestingly, there was a positive association between changes in condition and eosinophils counts (Table 5.2). Neither measure of helminth infection or condition was associated with changes in Th1 immunity measured as IFN-γ responsiveness (Table 5.2).

Helminth effects on microparasite infection

Forty percent of hosts were infected with coccidia at least once, and likelihood of coccidia of infection and coccidia abundance decreased with age (Table 5.1). Individuals from the LS herd were more likely to be infected than those from the CB or O herds (Table 5.1). Infection was least likely during the late dry season, and less likely during the early dry than late wet seasons. Infection intensity was also lower during the late dry season, compared to the early dry (Table 5.1). In models accounting for these host and capture variables as well as BCI and eosinophils, neither changes in Cf abundance, nor H abundance influenced the likelihood of becoming infected with coccidia (Cf: est = -0.0005, Z_{189} = -0.54, p = 0.59; H: est = -0.008, Z_{189} = -1.08, p = 0.28), remaining infected (Cf: est = 0.0001, Z_{106} = 0.09, p = 0.93; H: est = 0.009, Z_{106} = 1.1, p = 0.26), or the change in coccidia abundance (Cf: est = -0.0003, t_{269} = 0.09, p = 0.28; H: est = -0.002, Z_{269} = -0.93, p = 0.36). BCI also did not influence any aspect of coccidia infection (become infected: est = -0.12, Z_{189} = -0.51, p = 0.61; remain infected: est = -0.15, Z_{106} = -0.40, p = 0.69; abund = 0.090, Z_{269} = 1.1, p = 0.27). However, the change in eosinophils was positively correlated with a host's likelihood of remaining infected with coccidia (est = 0.067, Z_{106} = 2.68,

p = 0.007) and the change in intensity of coccidia infection (est = 0.015, Z_{269} = 2.60, p = 0.010; Fig. 5.2). By contrast, change in eosinophils did not predict whether a host would become infected with coccidia (est = 0.028, Z_{189} = 1.40, p = 0.16).

Effects on reproduction and survival

Because herd identity strongly influenced age at first reproduction ($F_{2,57}$ = 8.5, p < 0.001), with individuals in the CB becoming reproductive over 14 months later than individuals from the LS herd (Tukey test: p = 0.0004) and 7 months later than individuals from the O herd (Tukey test: p = 0.12), we examined the effects of helminths, condition, and immunity on age at first reproduction separately for CB versus LS and O. Surprisingly, in the LS-O herd, hosts with high Cf abundance at the previous capture were more likely to become reproductive for the first time at younger ages (est = -0.06, t_{22} = -3.2, p = 0.004), even when BCI was accounted for in the same model (est = -0.27, t_{22} = -0.06, p = 0.96). Conversely, H abundance did not influence age at first reproduction in the LS herd (est = -0.095, t_{22} = -0.47, p = 0.64). Within the CB herd, neither abundance of either helminth species nor BCI affected age at first reproduction (Cf: est = -0.011, t_{23} = -0.44, p = 0.67; H: est = 0.054, t_{23} = -0.52, p 0.61; BCI: est_{Cf} = -6.1, est_{H} = -5.5, t_{23}'s > -0.43, p's > 0.20). Among reproductively mature buffalo, the likelihood of being reproductive at a subsequent capture was not influenced by either Cf or H abundance (Cf: est = 0.00007, Z315 = 0.09, p = 0.93; H: est = -0.006, Z315 = -0.85, p = 0.40). Additionally, neither BCI nor eosinophils influenced the likelihood of being reproductive at the following capture (BCI: est = 0.065, Z_{315} = 0.27, p = 0.79; Eos: est = 0.018, Z_{315} = 0.97, p = 0.33).

Neither Cf nor H abundance had effects on host survival to a subsequent capture (Cf: est = 0.0004, Z_{616} = 0.49, p = 0.63; H: est = 0.005, Z_{616} = 0.89, p = 0.37). However, BCI was

positively associated with the likelihood of surviving to the subsequent capture (est = 0.52, Z_{616} = 2.12, p = 0.034). In addition, younger individuals had a marginally lower survival rate than older individuals (est = -0.013, Z_{616} = -1.90, p = 0.057). The predicted odds of survival based on age and BCI show that younger hosts in poor condition were least likely to survive to a subsequent capture (Fig. 5.3).

Discussion

The two helminths showed opposing relationships with host body condition , potentially indicating a direct cost of H infection and either a benefit of Cf infection or the positive influence of outside factors on both host condition and Cf abundance. Condition was positively associated with survival, so H infection could have negative, condition-mediated effects on survival, but Cf infection could indirectly enhance survival by increasing host condition. Individuals with higher Cf abundances were also more likely to become pregnant for the first time at a younger age than individuals with lower Cf abundances. Neither species had direct effects on microparasite infection, the likelihood of being reproductive at the subsequent capture, or survival, however they may influence host fitness indirectly via effects on immunity and condition. H directly affected host eosinophils and the degree of cross-regulation, both of which could influence microparasite susceptibly by altering Th1 defenses. Indeed, elevated eosinophils, an aspect of Th2 immunity, were associated with increases in coccidia abundance and failure to clear coccidia infection. Cf infection had no affect on host immunity or coccidia infection.

Hosts that gained H, from one capture to the next, lost up to a full body condition score, a substantial effect size given that mean BCI only varies by 0.3 between the wet and dry seasons. Conversely, hosts gaining Cf between captures could gain up to half a body condition score. For

H, this relationship could indicate a key cost of infection or that poor condition hosts are more susceptible to H infection. In support of the former interpretation, data from livestock show that H infection can cause emaciation, anemia, and mortality (Le Jambre 1995, Angulo-Cubillan et al. 2007, Thumbi et al. 2014). Moreover, in buffalo a previous cross-sectional study showed that H abundance was consistently negatively correlated with number of host physiological and condition parameters (Budischak et al. 2012). In terms of Cf, infection directly or indirectly improve buffalo condition, or unmeasured factors, such as foraging rate or host genetic background, could be positively influencing both host condition and Cf abundance. Although the effects of Cf on its hosts have not been measured previously, other *Cooperia* species typically cause only mild lesions in domestic livestock (Zajac 2006), but high intensity infections can decrease growth and milk production (Stromberg et al. 2012). Interestingly, experimental removal of the entire helminth community in another population of African buffalo resulted in increased body condition, suggesting that some aspect of helminth infection does indeed pose a condition cost to the host (Ezenwa et al. 2010). Thus the negative association between H and condition we describe here could be indicative of strong species-specific helminth effects on buffalo condition.

The two helminths also had different associations with host immunity. Hosts that gained H abundance also a greater skew toward Th2 immunity measured by the degree of cross-regulation of adaptive defenses (IL-4/IL-12 ratio). Similarly, in H-infected sheep, IL-4 concentrations were elevated for a week and T-helper cells, the main producers of IL-4, remained elevated for at least a month following infection (Robinson et al. 2010, Robinson et al. 2011). A skew toward Th2 responses could leave H-infected hosts more susceptible to microparasite infection (Cox 2001, Graham et al. 2007). Hosts that gained eosinophils had lower

H abundances and the successive capture, suggesting that eosinophils are effective at fighting H infection. Eosinophils are highly effective at combating helminth infection (reviewed in (Behm and Ovington 2000, Spencer and Weller 2010), including both *Haemonchus* (Balic et al. 2000, Ortolani et al. 2013) and *Cooperia* infections (Kanobana et al. 2002). Conversely, Cf infection was not associated with any aspect of host immunity.

While the abundance of neither helminth species was associated with coccidia infection, measured as changes in abundance, likelihood of clearance, and likelihood of becoming infected, we detected a strong association between eosinophil levels and the ability of the hosts to reduce or clear coccidia infection. If hosts elevate eosinophils to defend against H infection as proposed above, a reduction in host ability to defend against microparasite infection may be a direct cost of this strategy. This immune-mediated, positive association between H and coccidia also fits predictions of the Th1-Th2 tradeoff paradigm. In previous rodent deworming experiments, coccidia infection probability and intensity increased in dewormed hosts, and these patterns are consistent with a trade-off between helminth and coccidia defenses (Knowles et al. 2013, Pedersen and Antonovics 2013). Interestingly only one of the two helminths (H not Cf) may be involved in this interaction based on the helminth-immunity patterns we observed.

In addition to examining associations among buffalo physiology and GI parasite infection, we tested whether abundance of Cf or H, body condition, and Th2 immunity (eosinophils) affected reproduction. Surprisingly, Cf abundance was associated with earlier age at first reproduction in the LS herd. Age of first reproduction can greatly influence individual fitness in ungulates (Green and Rothstein 1991, Martin and Festa-Bianchet 2012). The positive relationship between Cf abundance and age at first reproduction could be related to the positive association between Cf and body condition detected in previous cross-sectional work (Budischak

et al. 2012) and this longitudinal study. Neither Cf nor H abundance influenced the likelihood of being reproductive at the subsequent capture. This lack of effect concurs with several previous studies where parasite infection reduced condition, but not subsequent reproduction (Stien et al. 2002, Newey et al. 2005, Ballesteros et al. 2012). Yet, in a parasite removal experiments in reindeer, infection negatively affected reproduction to a degree that could influence host population dynamics (Albon et al. 2002, Stien et al. 2002). Finer resolution of buffalo reproductive data that assesses reproductive success, calf survival, and calf quality may be needed to detect more subtle effects of helminth infection on host reproduction.

Although survival was not directly affected by Cf or H abundance, host condition was a key predictor of the likelihood of survival to a subsequent capture and the helminths had opposing associations with host condition. Consequently, H may have negative, condition-mediated effects on survival, but Cf infection may indirectly enhance survival by increasing host condition. Effects of condition on survival have been a detected in other wild ungulate populations (Stien et al. 2002, Wilson et al. 2004). However, direct effects of helminth infection on individual survival in mammals has only been documented in one previous field study of Soay sheep (Gulland 1992), while most longitudinal studies and removal experiments fail to detect survival effects (Stien et al. 2002, Newey et al. 2005, Ballesteros et al. 2012). Notably, the effect of helminth infection on survival of Soay sheep was detectable only during a severe population crash and within a class of hosts (males) that were more heavily infected with a pathogenic helminth species (Gulland 1992, Craig et al. 2006). Together, this study of Soay sheep and our species-specific condition data suggest that taking a species-specific approach may enhance the detectability of physiological and fitness costs helminth infection.

The two dominant helminth nematodes of buffalo had opposing associations with host

condition, and body condition was the main predictor of survival to the subsequent capture. Effects on host immunity and microparasite infection were also species-specific. H, but not Cf, was associated with a shift toward Th2 defenses and may indirectly influence microparasite infection, since coccidia infection was positively associated with innate Th2 defenses. We uncovered these intriguing patterns using a novel approach to track helminth species abundances through time in live, free-ranging hosts. Because H may impact individual survival and microparasite defenses, further research is warranted to explore the role of H infection in microparasite transmission and host populations, as well as to identify environmental and physiological drivers of H infection. The effects of helminth infection on host fitness may be cryptic and indirect, yet have substantial consequences for the dynamics of microparasites and host populations.

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Tables

Table 5.1. Estimates for the effects of herd (Lower Sabie [LS], Croc Bridge [CB, Other [O]), season captured (early wet [ew], late wet [lw], early dry [ed], late dry [ld]), and age in months on helminth infection. Sample size (n) indicates number of total and unique hosts. Significance is indicated by asterisks ($p < 0.05$*, $p < 0.01$**, $p < 0.001$***).

Parasite Infection	n	Herd	Season	Age
Likelihood of infection				
Infection status	778, 249	CB > LS ** LS > O *	ew > ed*** ew > lw* ld > ed * lw > ld ***	-0.084 *
Cf status	778, 249	CB > LS ** O > LS *	ew > ed **	-0.018 **
H status	778, 249	CB > LS *** CB > O *	ew > ed *** ld > ed ** ew > lw *	-0.021 ***
Coccidia status	778, 249	LS > CB ** LS > O *	ed > ld *** ew > ld * lw > ld *** lw > ed*	-0.0082 *
Infected Hosts				
Cf Intensity	294, 132			-0.00093
H Intensity	199, 116	CB > LS *** O > LS *		-0.0062 *
Coccidia Intensity	223, 133		ed > ld *	-0.0067 *

Table 5.2. Estimates of the relationships between changes in Cf and H abundance and changes in host immune function. Sample size (n) indicates number of total and unique hosts. Significance is indicated by asterisks ($p < 0.05$*, $p < 0.01$**, $p < 0.001$***).

Fixed Effects	**Δ Eos** n = 434, 123	**Δ IFN-γ** n = 384, 118	**Δ IL-4/IL-12** n = 100, 68
Herd			
Season Change	dw > dd * dd > wd ** dw > wd *** ww > wd **		
Age	-0.021	-0.0005	-0.0002
Interval	0.023**	0.0020**	-0.0013***
Δ Condition	0.95***	0.063	0.0067
Δ Cf Abund	-0.0006	0.0002	0.0001
Δ H Abund	-0.0034**	0.0011	0.0011*

Figure Legends

Figure 5.1. Diagram of the direct (black) and indirect (gray) pathways by which macroparasite infection may influence microparasite infection and host fitness.

Figure 5.2. Change in eosinophil count (Eos) was positively correlated with change in coccidia abundance (-2: large losses; -1: small losses; 0: no change; 1: small gains; 2: large gains).

Figure 5.3. Predicted odds of surviving to the next capture (~180 d) increased with body condition index (BCI) and age.

Figure 5.1

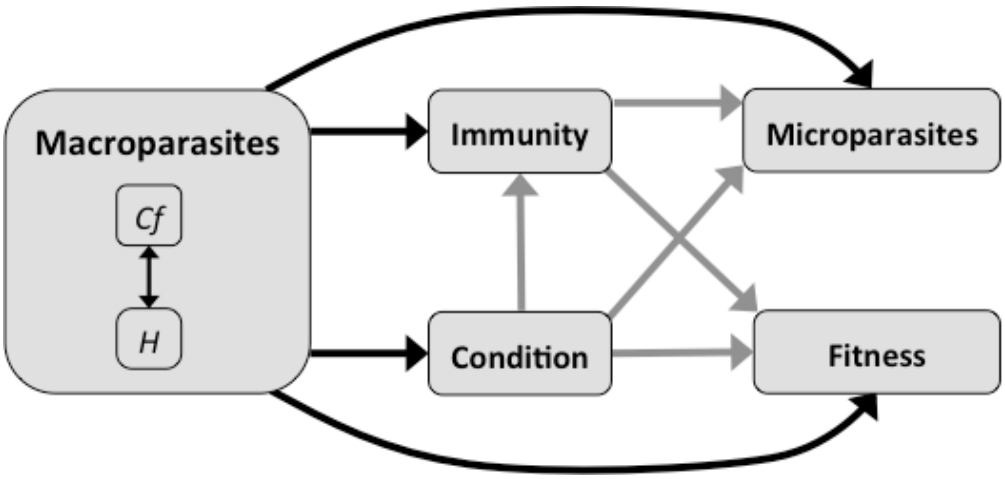

Macroparasites
Cf
H
Immunity
Microparasites
Condition
Fitness

Figure 5.2

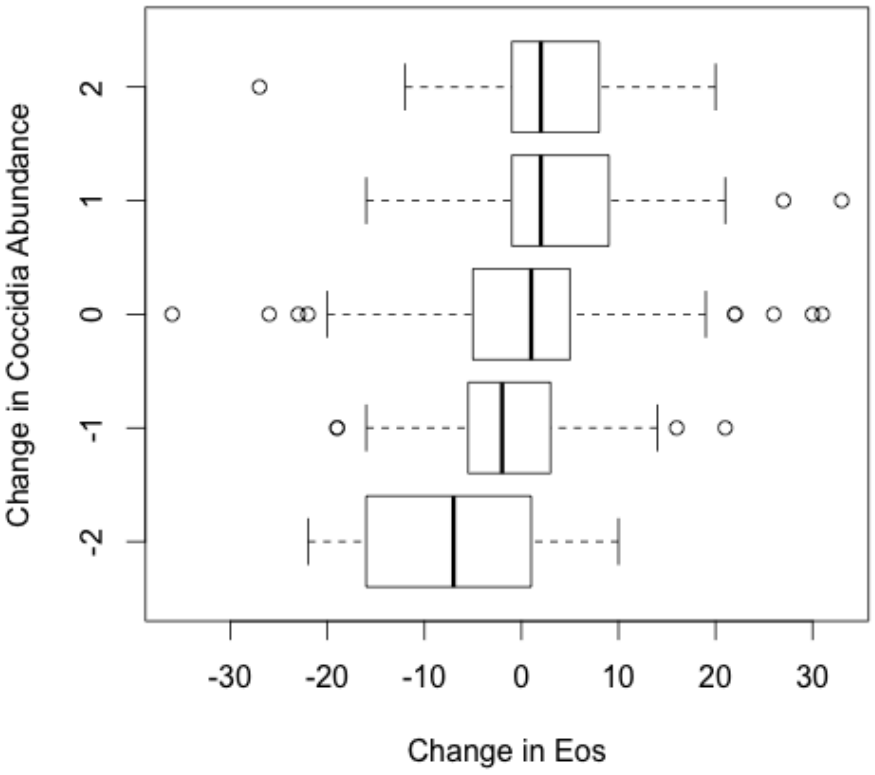
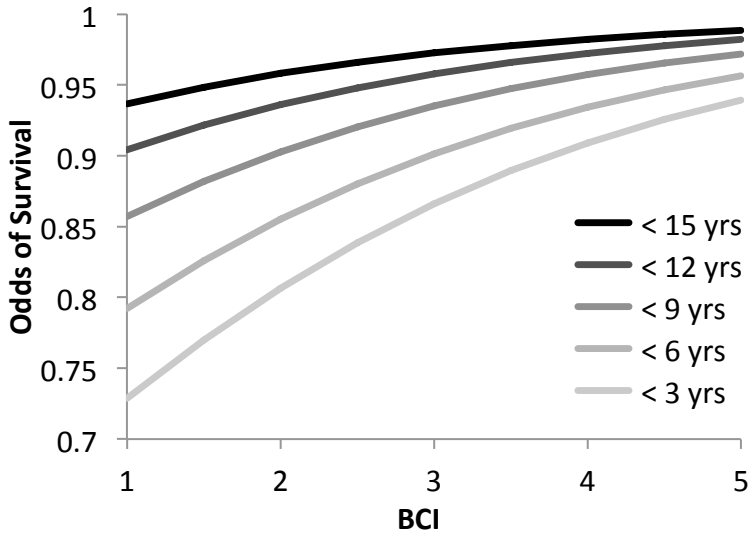

Change in Coccidia Abundance
2
1
0
-1
-2
-30
-20
-10
0
10
20
30
Change in Eos

Figure 5.3


1
0.95
0.9
0.85
0.8
0.75
0.7
Odds of Survival
1
2
3
4
5
BCI
< 15 yrs
< 12 yrs
< 9 yrs
< 6 yrs
< 3 yrs

CHAPTER 6

CONCLUSIONS AND FUTURE DIRECTIONS

The goal of this dissertation was to improve the mechanistic understanding of the causes and consequences of parasite coinfection. Immune- and resource-based mechanisms are difficult to discern in natural populations, and the effects of resources have largely been ignored in laboratory experiments. My dissertation research examines how host resources and immune function interact to influence parasite coinfection and host fitness, both in a wild population and experimentally, by pioneering the interdisciplinary application of community ecology, veterinary, and molecular parasitological tools to new questions. I will highlight, chapter by chapter, the contributions my dissertation research makes to our understanding of the factors that shape parasite community composition and the costs of coinfection.

In Chapter 2, I found that physiological performance indices can detect the short-term costs of parasitism in wild African buffalo populations. Specifically, host hematological profiles appear to be sensitive to variation in infection levels, with parasite species richness and identity largely driving observed direct effects. I detected more interactions between GI parasite infection and hematological parameters than with a traditional measure of energetic reserves, a body condition index based on fat storage. Hematological profiles may represent a useful tool for assessing the costs of GI parasite infection in wild populations.

Importantly, Chapter 2 shows that the detectability of both direct and indirect effects depended on the resolution of the parasite data. With aggregated parasite data, physiological

effects may only emerge when effect sizes are considerably large. My results also suggest that aggregated data can mask parasite effects because even closely related parasite species may alter host hematological parameters and immunity in different ways and to differing degrees. Although it is fairly well-established that different GI helminths have distinct effects on livestock hosts (Stear et al. 1998, Bowman 2009), there is still very little known about how individual species within the GI parasite community combine to contribute to the costs of infection in wildlife species. Determining helminth community composition and relative species abundance from fecal samples is a substantial challenge. My use of a combination of genetic-based identification and traditional egg counts revealed strong patterns that were not detectable with either data set alone. These results highlight a practical way in which traditional egg count data can be combined with molecular data to address key questions about helminth co-infection in wildlife populations where invasive sampling is not feasible.

In Chapter 3, I examined the structure of helminth communities by comparing community composition differed within control and anthelmintic-treated hosts after parasites were allowed to recolonize for 3-11 months. Disturbance by anthelmintic treatment influenced gastrointestinal helminth community composition and structure at both the among-host and within-host scales, allowing me to test key predictions about the forces shaping parasite communities. All but one community metric suggested that control communities are isolationist, while disturbed communities are interactive. While equilibrium parasite communities often appear to be randomly assembled, my data suggest that biotic interactions play an important role in the development of parasite communities following disturbance. Importantly, differences in structure between disturbed and equilibrium communities may account for some of the

inconsistencies regarding the role of biotic interactions in shaping parasite communities in wild populations.

Treated communities increasingly resembled control communities with time since disturbance, revealing an important temporal sequence where biotic interactions are important in community assembly following disturbance, but random factors play an increasingly important role over the course of succession. Temporal changes in community structure over time were largely driven by an increase in the intensity of a single dominant parasite species (*Cooperia fulleborni*), suggesting that treated communities preferentially accumulated Cf, rather than randomly accumulating species. Thus to fully understand the factors influencing parasite community structure, my research in Chapter 3 reveals that it is important to study all phases of community establishment, since equilibrium 'climax' communities may appear isolationist, but biotic interactions among parasites may direct community assembly.

My goal in Chapter 4 was to examine how resource limitation influences the outcomes of co-infection, and to explore potential underlying mechanisms for these outcomes. Using factorial combinations of two dietary protein levels and three parasites, I found that parasite fitness and host performance strongly depended on diet quality and the combination of co-infecting parasites. Low quality resources increased egg-shedding, and therefore transmission potential, of one helminth species, but decreased egg shedding of the other. Facultative interactions between the helminth species were stronger within hosts fed a high protein diet, but facultative interactions between one of the helminth species and a microparasite were stronger within hosts fed a low protein diet. Resource scarcity and helminth infection also frequently co-occur in human populations, and our data suggest those populations may also be more vulnerable to microparasitic infection.

While separate immune- and resource-mediated effects on co-infecting parasites have been detected in previous studies (Graham 2008), Chapter 4 is the first experiment to explicitly test their relative importance for parasite fitness. Structural equation analysis allowed me to determine that indirect, immune-mediated interactions had the strongest effects on helminth fecundity in our study. Coinfection has been identified as a factor that can potentially generate super-shedders (Stein 2011), and this experiment adds to the small number of studies empirically documenting such effects (Sherertz et al. 1996, Cattadori et al. 2008, Lass et al. 2013). Furthermore, I detected interesting differences in immune function, growth, and egg shedding that suggest resources influence whether infected hosts adopt a resistance or tolerance strategy. Overall, my findings from Chapter 4 highlight that the outcomes of co-infection are context-dependent for both parasites and hosts, and that resources are a key context that shapes the magnitude and direction of parasite interactions.

In Chapter 5, I examined the physiological and fitness costs of helminth infection using a longitudinal study of African buffalo. Using a novel approach to track helminth species abundances through time in a wildlife population, I was able to detect opposing patterns that would be masked, even in an experimental treatment context, if the species were pooled. The two helminths showed opposing relationships with host body condition index, potentially indicating a direct cost of *Haemonchus* infection and either a benefit of *Cooperia fulleborni* infection or the positive influence of outside factors on both host condition and *Cooperia fulleborni* abundance. Neither species had direct effects on microparasite infection, reproduction, or survival, however they may influence host fitness indirectly via effects on immunity and condition. Because body condition influenced eosinophils and, in turn, eosinophils influenced microparasite infection duration and abundance, both helminth species have the potential to indirectly influence

microparasite infection. *Haemonchus* directly affected host eosinophils and the degree of cross-regulation, both of which could influence microparasite susceptibly by altering Th1 defenses. Most interestingly, *Haemonchus* and *Cooperia fulleborni* could have opposing, indirect effects on survival because hosts in good body condition were Importantly, this work reveals the potential importance of cryptic and indirect effects of helminth infection on host fitness. more likely to survive to the subsequent capture.

Together, the field and laboratory components of my dissertation highlight the strong effects that parasites can have on each other and their hosts, but, as in free-living communities, context is king. However, measuring the intermediate mechanisms by which parasites and hosts interact, namely via resources and immune function, can provide insight into coinfection outcomes for hosts and parasites. My research identifies several directions where further research is needed to advance our understanding of the causes and consequences of coinfection. Importantly, my research validates some of the interdisciplinary tools needed to study these questions in wild populations. First, to understand the effects of helminth infection on host physiology, immunity, and fitness, future studies should focus on collecting species-specific longitudinal data. My research demonstrates that hematological analyses can assess host physiological costs of infection (Chapter 2) and validates the use of parasite molecular identification tools to sub-lethally collect helminth infection data (Chapter 3). Second, parasite addition experiments offer an untapped, potential method to explore the species-specific costs of parasite infection in the wild. Lastly, building from my findings in Chapter 4, the importance of resources during single- and coinfections should be tested in mesoscosm and field experiments. Follow-up investigations are merited because the conservation and management implications of the effects of resource availability on hosts and parasites are vast, particularly if resources

influence resistance versus tolerance responses in hosts. Finally, as the tools to measure immunity in non-model organisms improve, combining immunological data with the approaches outlined above will enable a mechanistic understanding of the causes and consequences of coinfection in the wild.

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