ABSTRACT

SANDERS, ANDREW JOSEPH. Stoichiometry and Infectious Disease: Linking Chemical Elements and Parasitic Interactions. (Under the direction of Dr. Brad Taylor).

Resource theft is a core characteristic of parasitism. However, we lack a conceptual framework for understanding nutrient theft by parasites that applies across biological levels of organization. In this dissertation, I explored the application of the ecological stoichiometry framework to parasitic interactions.

In chapter 1, I conceptualized the synthesis of ecological stoichiometry and disease ecology. This synthesis resulted in the development of several hypotheses and predictions regarding the relationship between elemental nutrients and host-parasite interactions. Chapter 1 shows that ecological stoichiometry is capable of expanding our understanding of host-parasite interactions, and complimenting other approaches to studying infectious disease such as population and community ecology, and molecular biology.

In chapter 2, I explored the role of host diet stoichiometry on parasite production by rearing *Myxobolus cerebralis* infected *Tubifex tubifex* on a wide gradient of diet carbon to phosphorus (C:P) stoichiometries. I found a strong effect of *T. tubifex* diet C:P on *M. cerebralis* spore production, and a hump shaped relationship indicative of a threshold elemental ratio. Further, I compared the results from the laboratory reared worms to wild caught worms and found that spore production by wild *T. tubifex* is characterized by a similar relationship over their shared range of fine benthic organic matter C:P. Chapter 2 shows that host diet stoichiometry can be an important influence on parasite reproduction, but also that the relationship between them is complex and sometimes nonlinear. The results of chapter 2 are the first example of a TER in parasite production.

Chapter 3 explores the role of parasitism in consumer driven nutrient recycling (CDNR). Previous studies on the effects of parasites on CDNR reported elevated rates of nitrogen (N) excretion by parasitized hosts, and decreased host excretion N:P. I tested if these were general traits of host-parasite interactions by measuring the effect of parasites on host excretion in five host-parasite pairs. I also investigated the relationship between host and parasite tissue stoichiometry and parasite effects on CDNR. I found that relationships were highly variable and interaction specific. Differences in results across host-parasite pairs and levels of parasite burden allowed me to develop hypotheses regarding their causal mechanisms.

Together, the first 3 chapters presented here develop and test a framework for understanding the role of elemental nutrients in host-parasite interactions, increase our knowledge and understanding of parasitic interactions, and open several new avenues for further inquiry. Chapters 2 and 3 demonstrate that the distribution and abundance of nutrients in ecosystems and the dynamics of host-parasite interactions are inexorably linked.

Stoichiometry and Infectious Disease: linking chemical elements and parasitic interactions

by
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APPROVED BY:

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DEDICATION

This dissertation is dedicated to my parents, Joe and Cathy Sanders

And to my wonderful and supportive partner, Rhiana Jones

And to the indomitable spirit of indigenous peoples around the globe.

BIOGRAPHY

I was born and raised in Tahlequah, Oklahoma, the older of two siblings by a year and a half. One of the strongest threads running through my childhood was scouting. The Boy Scouts of America helped foster in me a deep and personal relationship with the outdoors, as well as inculcating strong convictions regarding community and global citizenship, responsibility, respect, leadership, morals, ethics, duty, and personal independence. After graduating high school, I joined the Navy, where I further developed these parts of myself, especially in regards to global citizenship, leadership, honor, courage, and commitment. During my time in the Navy I traveled to 5 foreign countries, including South Africa, the United Arab Emirates, The United Kingdom, Germany, and the Kingdom of Bahrain. Such wide traveling substantially opened my mind and taught me a great deal about the commonalities of the human experience.

After honorably separating from the military, I attended college at the University of Arkansas on the Post-9/11 G.I. Bill. I majored in biology, a discipline I was able to widely explore in high school thanks to the dedicated efforts of my dear teacher Lisa Yahola. The generous terms of the G.I. Bill allowed me to support myself during college while working no more than 20 hours a week at a minimum wage job in an aquatic ecology laboratory on campus, the Evans-White lab. Dr. Evans-White and her graduate students welcomed me into the lab as a full member, included me in all aspects of the lab, professional and social, and spent much time and effort instructing me in the scientific method and many specific field and lab protocols. Only because of the privilege the G.I. bill afforded me, and the time and trust that Dr. Evans-White and her students gave to me, did I develop the solid foundation upon which all of my subsequent success in scientific research has been built.

While in college at the U. of A., and thanks to skills I developed in the Evans-White lab, I also had the opportunity to do ecological research in Costa Rica, during both the summer of 2012, through the NSF funded Native American and Pacific Islander Research Experience for Undergraduates, and in the fall of 2013 through the Organization for Tropical Studies semester abroad tropical ecology course. These experiences in the Central American jungle further helped me grow as a scientist, refine my research skills, and decide to attend graduate school. They also introduced me to my wonderful life partner, Rhiana Jones.

In 2014, after completing my bachelor's of science degree at the University of Arkansas, I started my PhD at Dartmouth College. I then transferred to North Carolina State University in 2015, with my advisor, Brad Taylor.

In my free time, I enjoy hunting, fishing, hiking, biking, climbing, paddling, camping, reading, wrenching, and the company of my friends; and preferably as many of those at the same time as possible.

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I thank my dissertation committee for supporting the development and execution of the research presented here. I especially thank my advisor Dr. Brad Taylor for fostering my intellectual growth, self-confidence, and independence. I admire Dr. Taylor's abilities to think deeply, develop big ideas, pursue expansive projects, work diligently, pay attention to detail, and communicate through scholarly writing. I am continually impressed by the breadth and depth with which he knows the scientific literature. Dr. Taylor's example will stand as a guide-star for the rest of my career. I also thank Drs. Kevin Gross, Jeff Hinshaw, Craig Layman, and Jay Levine for their intellectual generosity and dedication to my success.

I appreciate my labmates, Ramsa Chavez-Ulloa, Jared Balik, Elin Bink, Amanda DelVecchia, Mick Demi, Matt Ealy, Jackie Fitzgerald, Sam Jordt, Shaian Lashani, and Derek West for their friendship, support, assistance, and helpful comments and suggestions. I also extend my appreciation to my officemates, classmates, colleagues, mentors, and professors Elliott Barnhart, Michael Butler Brown, Checo Colon-Gaud, Zach Eddy, Braden Elliott, Ryan Emanuel, Cari Furiness, Jonathan Giacomini, Sara Giacomini, Debora Goedert, Lisa Kim, Lindsey Kimmerling, Brady Kohler, Tom Kraft, Courtney Larson, Aranzazu Lascurain, Britany Morgan, Beth Reinke, Christine Urbanowitz, Andy Vacca, and Chelsea Vario-Petrenko for the same.

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skills, gear loans, photography, party planning, fishing tips, and for sharing such special experiences in such a special place with me.

I also thank my dear friends Gina Calabrese, Lauren Carley, Becky Dalton, Sean Griffin, Hal Halvorson, Jacob Heiling, Lauren Kinter, A.J. Manship, Gabby Pardee, and Rachel Steward for all of the above and more. Your true and deep friendship deserves special recognition, I owe so much of my success to each of you and I will always remain your loyal friend. Thank you also to Dash Donnelly. We didn't get to enjoy each other's friendship for long in this life, but we will in the next. Find a good spot to put our duck blind in the meantime.

Thank you to Russell Hudgens, Lisa Yahola, and Michelle Evans-White for instilling in me a love of science generally, and biology and aquatic ecology specifically, for seeing your students' potential, and for being so dedicated to ensuring they received a quality education despite the challenges you faced.

I especially thank my thoughtful, caring partner Rhiana Jones, and my parents for their unconditional support and unwavering confidence in me.

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Forum

Using ecological stoichiometry to understand and predict infectious diseases

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A key characteristic of host–parasite interactions is the theft of host nutrients by the parasite, yet we lack a general framework for understanding and predicting the interplay of host and parasite nutrition that applies across biological levels of organization. The elemental nutrients (C, N, P, Fe, etc.), and ecological stoichiometry provide a framework for understanding host–parasite interactions and their relation to ecosystem functioning. Here we use the ecological stoichiometry framework to develop hypotheses and predictions regarding the relationship between elemental nutrients and host–parasite interactions. We predict that a suite of host and parasite traits, stoichiometric homeostasis, host diet stoichiometry, and biogeochemical cycling are related to disease dynamics, host immunity and resistance, and bacterial growth form determination. We show that ecological stoichiometry is capable of expanding our understanding of host–parasite interactions, and complementing other approaches such as population and community ecology, and molecular biology, for studying infectious diseases.

Keywords: stoichiometry, parasitism, infectious disease

Synthesis

The ecological stoichiometry framework is increasingly being applied to the study of host parasite interactions. In this manuscript we review past work on ecological stoichiometry and infectious disease and further synthesize the two fields by using stoichiometry to interpret the results of other infectious disease studies that have used non-stoichiometric nutrient currencies, and by using theory to develop new hypotheses and research avenues. We show that ecological stoichiometry provides a framework for uncovering general patterns and understanding the role of nutrients across the diversity of host–parasite interactions.

Parasites, resource theft and elemental nutrition

Parasites rely on their hosts to supply the resources necessary for growth and reproduction. As resource theft is a core characteristic of host–parasite interactions, an understanding of the flow of resources from host to parasite is central to understanding parasitic relationships (Smith et al. 2005). The availability of host nutrients to a parasite depends on two basic factors: the quantities of nutrients the host possesses in the parasitized tissues or organs (Fig. 1A), and the degree to which the host's nutrients are protected from parasites (Fig. 1B). Both factors are directly or indirectly related to nutrients the host obtains from its environment (Fig. 1C), or what may be referred to as host nutrition (Smith 2007). Thus, parasite nutrition is a function of host nutrition.

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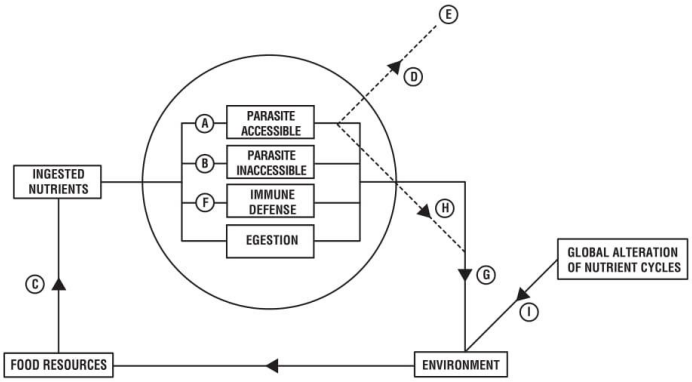

Figure 1. Hypothesized linkages among host–parasite interactions and ecological stoichiometry are represented by a generic host (large circle). (A) Ingested or assimilated host nutrients that are accessible to the parasite. (B) The portion of a host's dietary nutrients that are ingested or assimilated and not accessible to the parasite. (C) Elemental nutrients ingested by the host, or host diet. (D) Parasite growth, reproductive rate, and the number of infectious propagules emitted by the host. (E) Variability in parasite virulence. (F) Host immune function. (G) Host nutrient excretion rates and stoichiometry. (H) Parasite-induced changes in host excretion rates and stoichiometry. (I) Global alteration of nutrient cycling affects nutrient stoichiometry of food resources available to the host.

Although there are many studies exploring the role of host nutrition in infectious diseases, relatively few describe the relationships and feedbacks between specific nutrients and infection dynamics (Aalto et al. 2015). Past studies have used a variety of currencies for nutrition, from nutritional indices based on growth data (Hall et al. 2009), to macromolecules (Akpom and Warren 1975, Peck et al. 1992), to the chemical elements necessary for life (Pulkkinen et al. 2014). The last of these, the elemental nutrients (e.g. C, N, P, Fe ...), have two major advantages over other currencies: 1) they are universally necessary for all organisms, and 2) they cannot be chemically transformed from one element to another. Thus, elemental nutrients are relevant to both host and parasite, and can be traced from the environment to the host, from host to parasite, and back to the environment (Fig. 1).

Early reviews of the effects of nutrition on infectious disease focused on macromolecules. They concluded that parasites, like their hosts, could be limited by nutrients, and that the supply of nutrients available to the parasite from the host could influence disease dynamics and pathology (Smith et al. 2005, Smith 2007). More recent studies have begun to apply the stoichiometric framework to these questions. Recent reviews have focused on these stoichiometric studies (Aalto et al. 2015), and have reviewed the impact of infectious disease and parasitism on biogeochemical cycles, noting that the majority of studies have tested for effects of host density rather than trait mediated effects (Preston et al. 2016). These past reviews represent milestones toward understanding the interactions between nutrients and infectious disease, each outlining current knowledge to direct future research questions as well as integrate ecosystem and disease ecology. We intend to further develop these ideas and generate more explicit hypotheses by not only reviewing studies that have applied ecological stoichiometry, but also by applying the stoichiometric framework to studies that have used other currencies, and by incorporating first principles.

Here, our goal is to review host–parasite interactions within the stoichiometric framework, and to use the framework to develop hypotheses and predictions, without restriction to particular systems or taxa. The stoichiometric framework takes advantage of the properties of elemental nutrients to offer mechanistic understanding and explanatory predictions for host–parasite interactions that are generalizable across organisms and ecosystems. Our goal is to integrate disparate concepts from ecological stoichiometry and disease ecology to develop hypotheses and predictions that are explicitly mechanistic and that include stoichiometrically mediated changes in parasite and host traits.

Ecological stoichiometry

Ecological stoichiometry is a theoretical framework that describes balances and fluxes of chemical elements among organisms and between organisms and the environment (Sterner and Elser 2002). Because ecological stoichiometry focuses on the chemical elements necessary for all life, it is easily generalizable across systems and taxa. This is particularly useful in studying multi-trophic interactions such as infectious disease, where participants in the interaction require the same elemental nutrients but in different forms (e.g. organic versus inorganic). It also allows us to make generalizable predictions about the roles of specific elements in infectious diseases, and about linkages between infectious diseases and nutrient cycles, by uniting them with a common, multimetric currency. Further, the quantitative nature and simple assumptions (e.g. Liebig's law of the minimum; conservation of matter) of the stoichiometric approach lend it to mathematical modeling, which can then be integrated

with mathematical disease models (Vannatta and Minchella 2018).

A major advantage of ecological stoichiometry is that physiological and life-history traits of host and parasite can be explicitly linked to predictions about the influence of elements on host–parasite interactions. In this regard, ecological stoichiometry complements other approaches (e.g. population modeling) to studying host–parasite interactions. Knowledge of the host's and parasite's physiology and life history can be used to make inferences about its chemical make-up and nutritional demands based on the principles of ecological stoichiometry (Reiners 1986). The "growth rate hypothesis" (Elser et al. 1996) is a classic example: organisms with higher growth rates require a greater cellular concentration of ribosomes, and thus have greater tissue rRNA content. Hence, these organism's stoichiometric signature (tissue carbon:phosphorus) should be closer to that of phosphorus (P) rich RNA. As a consequence, organisms with higher growth rates have lower tissue carbon (C) to P and nitrogen (N) to P ratios, and higher P demand; hence, of the P that is consumed by faster growing organisms, less is egested or excreted as waste products (Elser et al. 1996, however, see Sardans et al. 2012). Thus, growth rate can be useful in predicting the P demands of both parasite and host even when direct measures of tissue chemistry are unknown. Similarly, body size, photosynthetic rate, cellular organelles, structural tissues (such as bone, chitin and cellulose), behavior, method of nutrient acquisition, and various other traits can all be used to help predict an organism's nutritional demands and fluxes (Allen and Gillooly 2009, Meunier et al. 2017). Thus, considering traits of both the host and parasite within an elemental stoichiometry framework offers an approach to making predictions and improving our understanding of the outcome of host–parasite interactions.

Elemental physiology of host and parasite

Ecological stoichiometry can be used to explicitly link the elemental physiology of host and parasite to predict host–parasite interactions. Clasen and Elser's (2007) study on Chlorella Virus is one of the few explicitly stoichiometric studies on the effects of host nutrition on infectious disease. A physiological trait germane to all viruses is that they have a lower C:P ratio than their hosts' cells due to their smaller size, high growth rate, and more simple cellular and mechanical structure (Jover et al. 2014). Thus, Clasen and Elser (2007) hypothesized that because the C:P ratio of Chlorella Virus is much lower than that of its host, the alga *Chlorella NC64A*, viral production would be suppressed when the alga was grown in low P versus high P media. This prediction was supported by experimental data showing that the number of viral particles produced per host cell was significantly higher for cells cultured in P rich media (Clasen and Elser 2007). Thus the stoichiometry of host resources was shown to influence the production of infectious propagules, which in turn would be expected to increase transmission pressure (Fig. 1D). Interestingly, the alga's growth and reproduction were similar in both media, suggesting that changes in nutrient availability that do not affect host growth and reproduction can still be relevant for host–parasite interactions. Phosphorus specific effects on viral disease are also supported by evidence that prevalence of barley and cereal yellow dwarf viruses in a grassland plant community increased in response to P fertilization but not N (Borer et al. 2010). The consistent enhancement of viruses by P more than N agrees with our biological understanding of viruses, which are essentially packages of nucleic acid contained within a protein envelope, and are extremely P rich compared to most host organisms (Elser et al. 1996, Clasen and Elser 2007, Jover et al. 2014). Thus, knowledge of parasite and host physiology in conjunction with a stoichiometric framework can generate explanatory hypotheses and predictions of host–parasite interactions in the natural environment.

Bacterial parasites, like viruses, are much smaller than their hosts, or their hosts' cells, and have much higher rates of growth and reproduction. Accordingly, they have relatively low C:P and N:P ratios by virtue of their high P content (Fagerbakke et al. 1996, Elser et al. 2003, Makino et al. 2003). Thus, bacteria are expected to be generally P limited, especially if their host is itself subsisting on P deplete resources. Published results demonstrate that bacterial production can be strongly influenced by the C:P of their host's diet, and that decreases in bacterial production related to high host diet C:P can lead to lower transmission and infection prevalence (Frost et al. 2008b).

Studies of fungal parasites of plants have shown that fungal sporulation and biomass are more strongly influenced by N than P (Jensen and Munk 1997), an outcome that is predicted based on the lower C:N ratio of fungi versus their plant hosts and is consistent with ecological stoichiometry. *Rhizophydium megarrhizum*, a chytrid parasite of Cyanobacteria, has been shown to increase zoospore production under elevated N:P conditions, and decrease under low N:P conditions (Frenken et al. 2017). Similar to the results of Clasen and Elser (2007) in their Chlorella Virus study, experimental N:P conditions had a much stronger effect on zoospore production than host growth rate. Fungal parasites of animals are also sensitive to N; *Batrachochytrium dendrobatidis* (Chytrid) growth is known to be influenced by media C:N (Piotrowski et al. 2004). Conversely, P limitation was shown to have no significant effect on spore production by a microsporidian parasite of *Daphnia* (Aalto and Pulkkinen 2013). P deplete media was shown to decrease spore production by a chytrid parasite of the alga *Astrionella*, relative to standard media, but the effect of P limitation on *Astrionella*'s growth was substantially more severe, suggesting that the pathogen was less sensitive to P availability than its host (Bruning and Ringelberg 1987). Shifts from N to P limitation are also associated with shifts from bacterial decomposition of cellulose to fungal decomposition, further suggesting that fungi are more sensitive to variation in N availability than P (Güsewell and Gessner 2009). Studies of soil communities have shown that P fertilization increases the flow of energy through bacterial pathways relative to fungal (Parfitt et al.

2010), and that increases in soil C:N, C:P and N:P over forest chronosequences were related to shifts in energy flow from bacterial pathways to fungal pathways (Williamson et al. 2005). Moreover, fungi usually have slower growth rates than bacteria. Hence, the finding that fungi dominate or perform well under low P conditions, or P limitation, is consistent with the growth rate hypothesis. Taken together, applying a stoichiometric approach to fungal diseases may open new discoveries linking variation in host or environmental nutrients to patterns in the emergence and distribution of fungal parasites, and better understanding and prediction of host–fungal interactions.

Helminths, which are much larger and slower growing than either bacteria or fungi, are expected to be even more sensitive to dietary N. Helminth fecundity has been shown to be density dependent (i.e. helminth fecundity is inversely related to worm burden) (Anderson and May 1985), suggesting intra-specific competition between individual co-infecting worms, potentially for nutrients. Evidence suggests that reducing carbohydrate in the host's diet (decreasing diet C:N) can alleviate the density-dependent constraints on helminth fecundity (Bundy and Golden 1987), and that increases in host diet % protein can increase the percentage of eggs released by an infected host which are viable (Akpom and Warren 1975). Increases in detrital %N have been shown to be related to increases in the number of cercaria produced by trematode infected amphibians (Stephens et al. 2016). Together, these results suggest that helminth transmission is sensitive to host diet C:N. There is evidence that trematode cercaria production can be influenced by host diet P content, but cercaria production was higher in both low and high P treatments relative to an intermediate control, suggesting that nutrient limitation of the parasite was not the causal mechanism (Narr and Krist 2015).

From viruses to microbes and metazoans, parasites respond to the stoichiometry of their hosts' diets. With this core principle in mind, we now look more closely at key concepts from ecological stoichiometry in the context of host–parasite interactions, hypotheses regarding interactions between stoichiometry and specific facets of host–parasite relationships, and broader ideas regarding the stoichiometric impacts of parasites on the ecological matrices they are imbedded within.

Stoichiometric homeostasis and host–parasite interactions: predictions

Homeostasis, the ability of an organism to maintain constant internal conditions, is a defining characteristic of life, and a key concept of ecological stoichiometry. Stoichiometric homeostasis is the ability of an organism to maintain constant body chemistry in the face of variable diet chemistry. There is substantial variation in the strength of stoichiometric homeostasis across taxa (Persson et al. 2010).

Weak stoichiometric homeostasis is the result of an organism's ability to store excess nutrients that have been consumed, or decrease their stores of nutrients that are in short supply (Meunier et al. 2014). Aalto et al. (2015) propose that weakly-homeostatic hosts may suffer less fitness impacts from elemental exploitation by parasites than strictly homeostatic hosts, because they may be more tolerant of parasite driven nutrient depletion. We expand on this hypothesis and suggest that the relationship between stoichiometric homeostasis and parasitism is more complex, and depends on the underlying homeostatic physiology of the host (Table 1). We pose two related hypotheses concerning the effect of weak stoichiometric homeostasis on host–parasite interactions (Fig. 2): 1) hosts that have weak stoichiometric homeostasis, and have readily accessible nutrient stores, become better or worse resources for the parasites depending on whether the host's stoichiometry shifts closer to, or farther from, the parasite's nutritional demand (Fig. 2 – solid line). For example, viruses have high P demand and thus a weakly homeostatic host consuming P rich food becomes more P rich in its body stoichiometry and, therefore, a more suitable host for replication of the virus. Conversely, if the host is feeding on P deplete food, the virus will experience exacerbated P limitation. Alternatively, 2) if stoichiometrically weak hosts store excess nutrients in an organ, organelle, or chemical form that is inaccessible to parasites, their diet stoichiometry should have less influence on their parasites (Fig. 2 – dashed line), and their parasites should be more host specific and specialized to access those stores.

Further, maintaining stoichiometric homeostasis, requires energy expenditure in accordance with the 1st law of thermodynamics (Sterner and Elser 2002). Therefore, parasites are predicted to negatively impact their hosts' ability to invest energy into regulating body chemistry, weakening host homeostasis (Table 1). Indeed, Frost et al. (2008a) showed that not only did bacterial infection alter the stoichiometry of *Daphnia* tissue, but that it interacted with food stoichiometry, suggesting that the tissue stoichiometry of infected *Daphnia* responds to changes in diet stoichiometry different than uninfected *Daphnia*. More specifically, Frost et al. (2008a) found that increases in diet C:P resulted in a stronger reduction in tissue %P for infected *Daphnia* feeding on low C:P diets. We predict that a host's stoichiometric homeostasis is related to the diversity of parasites it may support and variation in competence within and among host species. Further, we predict that infected hosts will display greater stoichiometric homeostatic variability than uninfected hosts, and thus that some of the variation in field measurements of stoichiometric homeostasis (Halvorson and Small 2016) is due to parasitism.

Threshold elemental ratios and host–parasite interactions: a prediction

Another ecological stoichiometry concept that is relevant to host–parasite interactions, but has been overlooked to our knowledge, is the "threshold elemental ratio" (TER) hypothesis (Boersma and Elser 2006). The TER hypothesis proposes that disposing of excess dietary nutrients imposes a growth cost on organisms, so that growth exhibits a hump-shaped relationship with diet stoichiometry, whereby growth is maximized on a diet with a particular stoichiometry, and reduced as diet stoichiometry departs from this optimum in

Table 1. Selected hypotheses, predictions, and approaches for testing the linkages between ecological stoichiometry and infectious disease.

Hypothesis	Predictions	Approach to test
Host metabolic pathways direct the flow of excess nutrients toward or away from parasites (Fig. 2)	There will be a stronger relationship between host diet and parasite performance if nutrients are routed through tissues accessible to the parasite	Compare the effects of host diet on the performance of parasites infecting plastic and homeostatic tissues or hosts. Do parasites of homeostatic and plastic tissues or hosts respond differently to changes in host diet stoichiometry?
Parasites alter the relationship between host diet and host tissue stoichiometry	Parasites will decrease the energy available to maintaining elemental homeostasis resulting in greater plasticity among infected hosts	Compare the stoichiometric plasticity of infected and uninfected hosts. Are infected hosts less homeostatic than uninfected hosts?
Parasites exhibit TERs (Fig. 3)	Parasite growth or reproduction/replication will display a hump shaped relationship with host diet stoichiometry	Quantify parasite performance over a range of host diet stoichiometries. Is there a single host diet stoichiometry that results in maximum parasite performance and what is the shape of this relationship?
Epidemiological outcomes depend on the degree of overlap between host and parasite TERs (Fig. 3)	The less overlap between host and parasite TER the stronger the impact host diet stoichiometry has on the parasite's production	Quantify parasite performance over a range of host diet stoichiometries of at least two parasites that may have different TERs (e.g. virus and fungi). Do endemic and epidemic parasites have TERs that are closer and farther from host TERs respectively?
Host diet influences parasite virulence (Fig. 1E)	High C:nutrient ratios will shift pathogenic bacteria toward biofilm associated growth forms	Quantify biofilm formation over a range of host diet C:P stoichiometry. Can pathogenic bacteria be induced to form biofilms by feeding their hosts a C rich diet?
Host diet influences host immunity (Fig. 1F)	Decreasing N:Fe availability will decrease mammal immune function by limiting the host's ability to protect Fe stores and produced immune cells	Quantify immune function and parasite virulence across a range of diet N:Fe ratios. Do low N:Fe diets result in more severe disease symptoms, transmission risk, and thus epidemics?
	1. High C:P and C:N diets will decrease immune function by limiting the host's ability to produce immune cells and proteins	1. Quantify immune function over a range of diet C:P and N:P ratios. Do hosts fed C rich diets have poor immune function?
	2. Hosts feeding on extreme diet stoichiometries (i.e. very high or very low) will have increased susceptibility to infection, especially by gut parasites	2. Analyze gut microbiome and ability to resist infection over a range of diet stoichiometries Are hosts fed extreme diets more likely to become infected?
Parasites alter the ratios and rates of nutrient recycling by hosts (Fig. 1G)	Parasites will decrease excretion rates of nutrients that comprise a greater proportion of parasite biomass than host biomass, relative to other nutrients	Compare nutrient excretion rates of parasitized and unparasitized hosts. Do parasites predictably alter host excretion stoichiometry?
Global changes in nutrient cycling affect host–parasite interactions (Fig. 1G,C)	Increasing N:P in the environment will favor helminth and fungal parasites and reduce prevalence of bacterial and viral parasites	Compare parasite community composition and taxon-specific performance under different nutrient regimes. Do exogenous changes in nutrients have predictable impacts on host-parasite interactions?

either direction (Sterner 1997, Boersma and Elser 2006). We hypothesize that parasites are subject to TERs (Table 1). Given the life history of most parasites: sessile, small size compared to host, continuous feeding, and r selected reproduction, we hypothesize that food quantity is not limiting for parasites, and so parasite TERs are expected to be consistent for a given host–parasite pair.

There is evidence of TERs for pest population growth. For example, aphid performance and population growth is maximal when foliar nitrogen content is intermediate (Zehnder and Hunter 2009). We predict that over a wide range of host diet stoichiometries, parasite performance (measured as either growth rate, reproduction, or propagules emitted by the host) will display a unimodal relationship similar to growth rate TERs (Fig. 3).

Aalto et al. (2015) hypothesize that the outcome of infection can be expected to depend on the overlap of stoichiometric demands of host and parasite. We propose that more specifically, the juxtaposition of host and parasite TERs determine the outcome of infection (Table 1). For instance, if the TER optima of host growth and parasite reproduction are similar (Fig. 3 – solid and dotted line, respectively), then disease risk is predicted to be low to moderate because high parasite growth or reproduction can be counteracted by high host resistance or tolerance if the host is feeding at its TER optimum (diet A). Alternatively, if the TER optima of host growth and parasite reproduction are different (Fig. 3 – solid and dashed line, respectively) and the host is feeding on a diet that is closer to the optimum for parasite reproduction (diet B) than to the

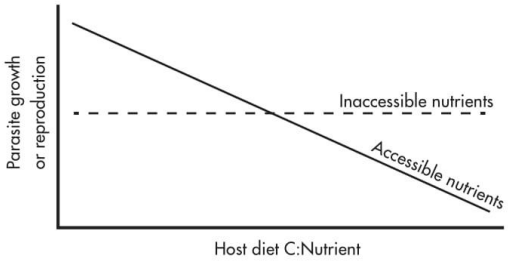

Figure 2. Hypothesized effects of homeostasis on the relationship between diet stoichiometry and parasite performance. Two alternative hypotheses for the relationship between host stoichiometric homeostasis and parasite production are presented. Solid and dashed lines represent two hosts both with weak stoichiometric homeostasis but that differ in how accessible their nutrients are to parasites. A host with weak stoichiometric homeostasis infected with a parasite that can readily access the host's nutrients becomes better or worse for a parasite depending on the stoichiometry of the host's diet (solid line). In contrast, parasite performance will be invariant if its host has weak stoichiometric homeostasis and the host's excess nutrients inaccessible to the parasite. For strongly homeostatic hosts, the same logic applies. If excess nutrients are processed in a way that makes them accessible to a parasite, then parasite performance should vary with host diet.

host's TER optimum, then disease risk is predicted to be at its highest, either because parasite production is high, the host cannot mount strong immune responses, or both. However, if the host is feeding at its TER optimum (diet A), then disease risk is predicted to be low. To our knowledge, no studies have tested for a TER in parasite growth rate or reproduction, or for interactions between parasite and host TERs.

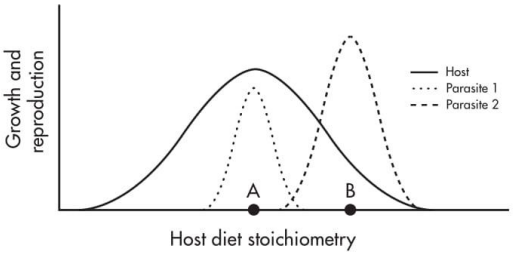

Figure 3. Some hypothesized associations between threshold elemental ratios (TERs) of a host and parasite. For instance, a host population feeding on diet A is predicted to have moderate risk to parasite 1 because diet A should be the optimum diet for host performance and thus immune function, which should reduce parasite performance. In contrast, a host population feeding on diet A is predicted to have low disease risk to parasite 2 because diet A is the optimum for host performance but very low quality for parasite 2. However, hosts feeding on diet B are predicted to have high disease risk to parasite 2 but not parasite 1 because diet B is the optimum diet for parasite 2 performance and very low quality for parasite 1.

Results have shown that snails infected with trematodes produce few infectious propagules on diets at the snail's $TER_{C:P}$, whereas they produce the most propagules on diets with high P, and intermediate numbers of propagules on low P diets (Narr and Krist 2015), suggesting that the parasite optimum is not the same as the host's diet stoichiometry optimum, supporting our prediction in Fig. 3. Additionally, in laboratory culture the maximum production of zoospores by the fungal parasite *B. dendrobatidis* occurs on diets with intermediate C:N ratios (Piotrowski et al. 2004). Mice infected with Schistosomiasis showed a hump shaped relationship in egg production with diet C:N, but the percentage of viable eggs decreased with increasing C:N (Akpom and Warren 1975). Thus, TERs are probably important to host–parasite interactions by affecting host and parasite performance differentially. We propose that non-linear relationships between host diet and parasite production can be explained by the relative TERs of host and parasite.

Stoichiometric effects on pathogenicity and virulence

Many organisms display changes in their phenotype or ontogeny when confronted with nutrient limitation. We hypothesize that parasites exposed to elevated nutrient availability will invest more heavily in pathogenic machinery or in outfitting propagules/offspring with greater stores of nutrients (Table 1). For example, many pathogenic bacteria have two distinct modes of growth: planktonic and biofilm associated. These two growth modes can depend on the relative amounts of different nutrients available to the bacteria (Cowan et al. 1991, Danhorn et al. 2004, Thompson et al. 2006, Monds et al. 2007), and differ in their pathogenicity (Parsek and Singh 2003). In the biofilm associated growth form, invasion and motility machinery are not expressed, and bacteria that can cause severe and acutely pathogenic infections in their planktonic form may persist avirulently in a host, or the environment, for decades in the biofilm associated form (Parsek and Singh 2003).

Biofilms confer numerous advantages to bacteria, including protecting them from immune system detection and attack, and from antibiotics (Fux et al. 2005). Because of their high sugar content, we predict that biofilms have high C:N and C:P relative to bacterial cells and host tissue, and thus biofilm production requires high C availability to the bacteria. When N, P, Fe or other non-carbon elements are limiting, the nutritional advantage of producing a biofilm matrix is twofold: It aligns demand with excess C availability, and it creates a complex organic structure that increases local nutrient availability by capturing materials that can then be degraded with extracellular bacterial enzymes (Wingender et al. 1999, Battin et al. 2003). Thus, shifting between growth forms enables bacteria to balance nutrient demand and availability.

The hypothesis that the availability of dissolved nutrients is related to bacterial growth form determination is not new, however, we propose that ecological stoichiometry represents a framework for understanding this interaction. Further, we predict that biofilms are the preferred growth form of pathogenic bacteria when sugars and other forms of carbon are

available in great excess relative to other potentially limiting nutrients, and published results support this prediction (Cowan et al. 1991, Danhorn et al. 2004, Thompson et al. 2006). For both pathogenic and non-pathogenic bacteria, expression of the Pho regulon, which is activated in response to P limitation, is related to biofilm formation (Danhorn et al. 2004, Monds et al. 2007). Thus, we hypothesize that nutrient effects on biofilm formation can be better understood using the ecological stoichiometric framework.

Fungal parasites have also shown phenotypic plasticity in the face of nutrient limitation. Chytrid parasites of cyanobacteria have been shown to produce fewer and larger zoospores under low N:P conditions, and many, smaller zoospores under high N:P conditions (Frenken et al. 2017). It is presumed that larger zoospores are outfitted with larger stores of lipid and fatty acid, allowing them to survive longer in the environment. It is also presumed that larger, more C rich zoospores take longer to produce. Thus, we hypothesize that these results are more indicative of C:N availability to the pathogen than N:P; as N limitation and C availability increases, the fungus balances demand, and slow growth rate under N limitation, by producing fewer zoospores of higher quality. Indeed, the authors note relatively higher light availability in the low N:P treatment that would be expected to increase photosynthetic rates and therefore cyanobacteria %C.

For parasites with more complex life cycles, we predict that host diet mediated nutrient availability will impact provisioning of infectious propagules. We predict that parasites metamorphosing from a parasitic larval stage to a free-living (non-parasitic) adult stage will display sensitivity to host diet %N in size at emergence and development time to adult emergence. In contrast, we predict that parasites with parasitic adults will show sensitivity to host diet %N and %P in egg viability and larval survival. For instance, increased % protein (assumed to reduce diet C:N), in bovine diets has been shown to result in female ticks that were larger at time of detachment, and somewhat shorter development time (time until detachment) (Gladney et al. 1973). Foliar %N has been shown to be positively related to the adult body weight of ichneumonid wasps that emerge from their herbivorous caterpillar host, and the ratio of emerging females to male wasps (Fox et al. 1990). Studies on the impacts of carbohydrate and protein on helminth parasites in the mouse model system suggest that the viability of worm eggs is negatively correlated with host diet C:N (Akpom and Warren 1975). These results mirror those of Urabe and Sterner (2001), who found that *Daphnia* reared on high C:P and C:N diets produced a substantially higher fraction of eggs that were non-viable than *Daphnia* reared on P and N replete media.

Effects of elemental stoichiometry on host immune function

In addition to the benefits afforded to parasite growth and virulence, a host diet rich in particular nutrients may benefit host immune function (Table 1), thereby circumventing the positive effects of nutrients on parasites, especially if the host can allocate nutrients to immune defense before they are stolen by its parasite (Fig. 1F, 1B). For example, iron (Fe) is a critical nutrient to all life as it plays an important role in respiration. Biologically available reduced Fe is scarce, and hosts protect their stores by binding it to large proteins such as transferrin and ferritin. Therefore, we predict that host diets with high N:Fe ratios support greater immune function because N-rich diets support the production of Fe binding proteins and their upregulation in response to immune challenge. This prediction is supported by evidence that humans suffering from kwashiorkor (protein deficiency) and hemochromatosis (a hereditary disease causing elevated serum Fe levels) have increased susceptibility to infectious disease (McFarlan et al. 1970, Muench 1989, Doherty 2007).

Dietary P has also been shown to influence host immunity. Evidence suggests that high P diets support cell mediated immunity, but the underlying mechanisms are unclear (Kegley et al. 2001, Heyer et al. 2015). It has also been shown that low dietary P can fail to support a stable intestinal microbiome, increasing the chance of invasion by pathogenic microbes (Heyer et al. 2015). Therefore, we predict that there is probably an ideal stoichiometric ratio of dietary nutrients (a TER) that supports a robust internal microbe community, and that diets imbalanced in nutrients for a healthy microbe community will predispose the host to invasion by gut microparasites that are better competitors for limiting nutrients. Taken together, we can use ecological stoichiometry to predict that hosts with diet C:N and C:P ratios higher than optimal should be at greater risk of infection because of the decrease in magnitude and rate at which immunological cells and proteins are produced, and that hosts with extreme diet nutrient ratios in general should be at greater risk of infection due to their microbiome's greater susceptibility to invasion.

Parasite effects on the stoichiometry of consumer-driven nutrient recycling

Consumer-driven nutrient recycling (CDNR, Fig. 1G), the re-mineralization of nutrients by living organisms, can be a major driver of local nutrient cycles (Vanni 2002, Small et al. 2011) and nutrient limitation (Elser et al. 1988). CDNR follows directly from ecological stoichiometry: if a consumer is stoichiometrically homeostatic to any degree, and their diet is variable and stoichiometrically unbalanced, then their waste products must vary with their diet to satisfy the law of conservation of mass (Olsen et al. 1986, Moody et al. 2015). Alternatively, if an organism undergoes physiological changes in response to ontogenetic or environmental shifts, but its diet remains relatively constant, a commensurate change in waste stoichiometry is expected (Dalton and Flecker 2014). Together, host and parasite are expected to have different nutritional requirements than the host alone. For one reason, parasites can cause physiological changes within the host (e.g. immune response) that affect nutrient retention by the host. Further, the parasite itself may have substantially different tissue chemistry from the host, and may constitute a

large proportion of the total biomass of the host and parasite combined, or may have much higher metabolic rates, or very high turnover rates. Thus parasites are predicted to alter the absolute rates of nutrient excretion, and often the relative rates (i.e. stoichiometric ratios; Fig. 1H, Table 1). The effects of parasites on CDNR have important implications for understanding and predicting the direct effects of consumers on nutrient cycling, yet studies of disease-induced changes in consumer functional roles, such as nutrient recycling, are rare.

Parasites should have higher P demand than their hosts, because parasites are usually smaller than their hosts and P demand increases as body size decreases (Elser et al. 1996, Woods et al. 2004, Allen and Gillooly 2009, Back and King 2013). Thus, parasites should decrease P in host excretion relative to N, as the P demand of the host and parasite combined should be greater than the P demand of the host alone, assuming parasitism does not disproportionately reduce growth demand for P of the parasitzed host. Indeed, snails infected with trematodes had higher N:P in their excretion than uninfected snails (Bernot 2013), supporting the hypothesis that parasites alter the stoichiometry of CDNR, and models suggest parasite-induced changes in excretion can alter N cycling at the ecocsystem level (Mischler et al. 2016). Further, *Daphnia* infected with bacteria and fungi excreted more N and P, and higher N:P (Narr and Frost 2016). This finding combined with our knowledge of how CDNR can affect nutrient limitation (Sterner 1986, Hall et al. 2003, Small et al. 2011, Munshaw et al. 2013) and shift the limiting nutrient (Sterner et al. 1992), leads to the hypothesis that parasites could shift ecosystem nutrient limitation by changing CDNR as has been shown for top predators (Elser and Carpenter 1988, Elser et al. 1988, Munshaw et al. 2013). Moreover, if parasites are capable of shifting nutrient limitation via CDNR by their host, then we predict that there will be feedbacks whereby a parasite-mediated shift in the identity of the limiting element (e.g. N to P or Fe) leads to repression of the parasite as that element becomes limited within their hosts (Fig. 1H, C). For examples, if an extremely P hungry parasite causes a severe reduction in its host's P remineralization resulting in exacerbated P limitation in the local environment, then the parasite will eventually experience increased P limitation itself.

Effects of global alterations in nutrient cycles on the relationship between stoichiometry and infectious disease

One of the primary strengths of ecological stoichiometry is the ability to apply it across biological levels from cells to ecosystems to understand the impacts of elements (Elser et al. 2000). Hence, ecological stoichiometry can be used for predicting and investigating how anthropogenic alterations to global nutrient cycles affect infectious disease through host–parasite stoichiometry (Fig. 1I). While P enrichment generally originates from relatively local sources (Ansari et al. 2011), human activities are increasing the availability of N at a global scale through atmospheric deposition, fertilizer runoff, and other pathways (Vitousek et al. 1997, Bergstrom and Jansson 2006). Further, efforts to mitigate nutrient pollution in lakes and coastal waters have generally resulted in greater decreases in P loading than N loading (Paerl 2009).

Increases in global N availability relative to P have been shown to shift a diversity of ecosystems to P limitation (Elser et al. 2009), or increase the strength of existing P limitation of primary producers and consumers (Bergstrom and Jansson 2006, Elser et al. 2010, Crowley et al. 2012, Penuelas et al. 2012). N pollution can also alter community structure by shifting the relative abundance and density of primary and secondary host taxa (Johnson and Chase 2004, Johnson et al. 2007, Bobbink et al. 2010, Aalto et al. 2015). Eutrophication by either N or P can also have a negative effect on biodiversity by altering species richness and evenness, which are known to modulate disease risk (Tilman et al. 2001, Johnson et al. 2013). The ability to trace the effects of changes in nutrient cycling from the base of food webs (i.e. plants, bacteria, fungi and algae) to higher consumers and their parasites is a powerful application of ecological stoichiometry for predicting and managing infectious diseases.

Using the principles of ecological stoichiometry, we can predict that increasing global N availability relative to P should shift nutrient limitation to P, or increase existing P limitation, for both hosts and their parasites (Table 1). Thus one potential consequence of N enrichment for parasites limited by P, such as bacteria and viruses, is that they should have lower performance or prevalence in P limited hosts, whereas parasites more sensitive to N, such as fungi and helminths, should have higher performance and prevalence, all else being equal. For example, *B. dendrobatidis*, the fungal agent that causes chytridiomycosis in amphibians, lives in the keratinized layers of the epidermis and feeds on keratin. If infected amphibians are feeding on N enriched food resources and are capable of investing greater N to replenish keratin, we predict that this will lead to faster growth and increased zoospore production by the fungal parasite, and higher prevalence and mortality within the host population. P limitation can also affect competition between co-infecting parasites (Lacroix et al. 2014) potentially leading to proliferation of previously rare parasites that are poor competitors under historical nutrient limitation scenarios. Further, evidence suggests that consumers evolving under eutrophic conditions can become increasingly sensitive to poor diet quality, and show stronger relationships between host diet quality and parasite virulence (Reyserhove et al. 2017). This suggests that anthropogenic changes in resource stoichiometry over evolutionary timescales will feedback, with eutrophication causing increasingly severe responses of infectious diseases to nutrient pollution. We hypothesize that because eutrophication decreases selection pressure on nutrient use efficiency, these results are likely generalizable, however further research is needed. For example, it is unknown if the results of Reyserhove et al.'s (2017) study were due to a decrease in the host's ability to compete for limiting nutrients, or the host's inability to meet immune system demand. We suspect the former, and that it

would be detectible as an increase in the parasite's production efficiency (production of infectious propagules relative to production of host tissue) as host diet C:P or N:P increased. In other words, if increased nutrient limitation reduced host growth more than it reduced parasite production, than that would suggest that the observed increase in virulence was due to the parasite outcompeting the host for nutrients, whereas an equal or lesser reduction in host growth compared to parasite production would suggest that the hosts diet is of too poor of quality to support immune function. We also suggest performing similar feeding experiments on a variety of other host–parasite pairings to better understand how host resistance and parasite virulence may evolve under different nutrient regimes. For example, by taking hosts (or parasite strains) from distinctly separate populations that have experienced widely divergent nutrient regimes for many generations and exposing them to a parasite strain (or host) isolated from a population that has been evolving under an intermediate nutrient regime.

We predict that shifts in the availability of different nutrients, and differential responses of various parasites to those shifts, should change the composition and relative abundance of the parasite community (Table 1). We also predict that bottom up effects on parasites will strengthen as anthropogenic eutrophication continues. Altogether, our current understanding suggests that the effects of eutrophication on host-pathogen interactions are the result of complex interactions between effects on the density of hosts, competition for nutrients between hosts and parasites, and competition for nutrients between co-infecting parasites. We predict that anthropogenic changes to biogeochemical cycling will promote global increases in fungal infection, such as chytrid, and lead to the emergence of previously rare infectious diseases. We also predict that global N enrichment will favor biofilm formation by pathogenic bacteria due to increased strength of P limitation. Most importantly, we suggest that ecological stoichiometry presents a powerful framework for understanding and predicting the impacts of human alterations to global biogeochemical cycling on emerging infectious diseases.

Conclusions

The theft of resources, principally nutrients, is an integral facet of host–parasite interactions. Ecological stoichiometry provides a framework for understanding fluxes of nutrients among organisms and between organisms and their environments, and thus offers substantial utility for studying infectious diseases. Here, one of our goals was to describe the application of ecological stoichiometry to infectious disease by presenting specific hypotheses and predictions.

Although focusing on the stoichiometry of chemical elements may be viewed as overly reductionist, we contend that ecological stoichiometry is a complement to, not a replacement for, other theoretical and empirical lines of inquiry. For example, macromolecules as a nutritional currency may provide precise predictions for specific host–parasite interactions because a given host or parasite's physiological requirements for them are often highly specific and well understood, however, they cannot be traced through food webs and the environment like the chemical elements. The stoichiometric approach offers greater generality and the inclusion of food webs and biogeochemical cycles into predictions of host–parasite interactions. Importantly, it provides a framework for uncovering general patterns occurring among the myriad of host–parasite interactions, and for integrating disease and ecosystem ecology (Aalto et al. 2015, Preston et al. 2016). However, a stoichiometric approach cannot address emergent properties of compounds, such as pheromones, medicinal secondary chemicals, or digestibility that are not readily apparent from their stoichiometry. Further, many aspects of host–parasite interactions cannot be addressed by nutritional studies alone – genetics, population ecology, and community ecology are all needed. Ecological stoichiometry is thus no panacea, but a potentially underutilized tool in the study of host–parasite interactions that can complement other approaches and explain additional variation. As a tool for simplifying the role of nutrition in biological processes, deviations between observations and predictions from ecological stoichiometry can highlight opportunities for discovery (Urabe et al. 2018). In many cases, ecological stoichiometry can be easily integrated into studies by taking some additional measurements of host, diet and parasite elemental content, as opposed to radically altering the scientific approach or study design. Thus we urge investigators to consider adding a stoichiometric dimension to studies of infectious diseases.

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Author contributions – The first and second author contributed equally to this manuscript.

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CHAPTER 2: host diet stoichiometry and threshold elemental ratios influence parasite reproduction

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ABSTRACT

A growing body of work has shown that parasites' growth and reproduction can be influenced by the food quality available to their hosts. Consumers, including parasites, require a balanced diet to grow. Unlike many consumers, however, parasites are beholden to the diet quality presented to them by their host, which is in turn a function of the host's own diet. Here, we tested the hypothesis that the relationship between parasite reproduction and host diet quality is subject to a threshold elemental ratio: that there is a balanced host diet that confers maximum growth to the parasite. We present results from a laboratory experiment where we fed a wide range of experimental diets to *Tubifex tubifex* infected with *Myxobolus cerebralis* and observed a hump-shaped relationship between *M. cerebralis* spore production and *T. tubifex* diet stoichiometry, indicative of a threshold elemental ratio. Further we compared these results to *M. cerebralis* spore production by wild *T. tubifex*, and found that spore production in the wild *T. tubifex* was characterized by a linear relationship with *T. tubifex* diet. Together, our results show

that host diet stoichiometry can be an important influence on parasite reproduction, but that the relationship between them is complex and sometimes nonlinear.

INTRODUCTION

True parasites and hemiparasites rely on their host to supply them with nutrients. While this strategy may be energetically favorable, it restricts the parasite's ability to optimally forage. Once an individual parasite has established a relationship with a host, they are then beholden to whatever resource quality that host presents them with. Thus, understanding the factors that influence the flux of energy and nutrients from host to parasite, and the sensitivity of parasites to variation in that flux, are important to understanding the nature of interspecific interactions characterized by resource theft (Smith et al. 2005).

One factor that likely influences the resource quality available to a parasite is the resource quality available to the host (Smith 2007). Previous studies have shown that parasite production, usually measured as production of infectious propagules, can be a function of the nutrient content of the host's diet. For example, algal cells grown in phosphorus (P) replete media produced greater numbers of viral particles upon bursting than those grown in P deplete media (Clasen et al. 2007). Similarly, powdery mildew infecting barley produced more spores in plots fertilized with nitrogen (N) (Jensen et al. 1997). Fertilization has been shown to increase the prevalence of viral infection in grassland communities as well (Borer et al. 2010).

Parasites of consumers are also sensitive to the nutrient contents of their hosts' diets: *Daphnia* infected with a bacterium produced more bacterial spores when fed P rich diets (Frost et al. 2008), Trematodes infecting mice produced more viable eggs when the mice were fed higher protein diets (Akpom et al. 1975), and trematodes infecting snails had higher cercarial production rates when snails were fed diets with lower N:P ratios (Bernot 2013). These studies use different currencies and methods, but generally support the hypothesis that the resource quality available to the host has implications for the parasite's production and fitness.

While evidence accumulates demonstrating that parasite sensitivity to host nutrition is widespread, much work remains in refining our understanding of this interaction and developing our ability to predict how changes in host diet quality will influence infectious diseases. In this study, we employed the ecological stoichiometry framework to deepen our understanding of the relationship between host diet and parasite production. Ecological stoichiometry is a framework that describes balances and fluxes of the chemical elements among organisms and between organisms and the environment (Sterner et al. 2002). Because ecological stoichiometry focuses on the chemical elements, it is especially useful for studying multitrophic interactions and placing biotic interactions in a broader biogeochemical context.

Threshold elemental ratios (TERs) are a theoretical response of consumers to diet quality that has emerged from ecological stoichiometry. The TER hypothesis predicts that consumers display maximum growth or production on a diet with some ideal stoichiometric ratio, and that production is reduced as diet stoichiometry moves away from that ratio in any direction, due to metabolic costs associated with processing excess nutrients (Boersma et al. 2006). Threshold elemental ratios have been observed in other kinds of consumers, including detritivorous insects and various fishes (Benstead et al. 2014, Halvorson et al. 2015), but not previously in parasites. In this study, we tested the hypothesis that parasites are sensitive to their host's diet quality, and that they should exhibit TERs in respect to host diet stoichiometry. We also tested the hypothesis that highly controlled laboratory studies may exaggerate or distort the influence of host diet on parasite performance and epidemic dynamics, as host nutrition is just one of many factors present in natural systems.

The myxozoan parasite *Myxobolus cerebralis* causes whirling disease in salmonid fishes (Sarker et al. 2015). *M. cerebralis* is a microscopic endoparasite with a complex, multi-host life

cycle. Myxospores are ingested by the benthic oligochaete *Tubifex tubifex*, which in turn produce actinospores (from here on "spores") that are infectious to salmonids. Upon death, infected fish release myxospores, completing the life cycle. *M. cerebralis* is invasive in North America and is highly fatal to native species of trout, particularly rainbow trout (*Onchorryncus mykiss*) and cutthroat trout (*Onchorryncus clarkii*) (Hedrick et al. 1999, Blazer et al. 2004). *Tubifex tubifex*, the primary host, is found throughout North America in both lentic and lotic habitats, and in pristine waters as well as those that are highly impacted (Zendt et al. 2000). In this study, we focused on the production of spores by infected *T. tubifex*. *M. cerebralis* is much smaller and faster growing than *T. tubifex*. The growth rate hypothesis suggests that smaller and faster growing organisms have high P requirements (Elser et al. 1996). Further, *M. cerebralis* spores primarily consist of nucleic acid rich sporoplasm (Sarker et al. 2015). Therefore, we hypothesized that *M. cerebralis* is P hungry relative to its host, and chose to focus on Carbon (C) to P ratios for this study. Carbon is commonly used as a reference element due to its ubiquity in living tissue.

METHODS

Experimental diet study

For the first phase of the experiment, we exposed *T. tubifex* to *M. cerebralis* myxospores at a dose of 50 myxospores per worm. All worms were exposed to the myxospores together, *en masse*. We then transferred the worms to 5 different experimental diets ranging from C:P = 35 to C:P = 1464. The experimental diets fed to the worms consisted of spirulina, Tetramin, Algamac 2000, bone meal, and agar combined in various ratios (table 2.1). We chose this range of diet stoichiometries to encompass roughly an order of magnitude of difference from the intermediate

diet (C:P = 134), which has been used in previous studies of this host-parasite interaction because it yields worm growth and parasite reproduction (Nehring et al. 2015). N:P and C:N stoichiometry also varied across diets similarly to C:P, though over much narrower ranges (N:P=6-29, C:N=6-53).

To avoid confounding effects of diet stoichiometry on resistance to infection, we reared all *T. tubifex* on the intermediate diet (C:P =134) during the 2 week period of exposure to *M. cerebralis* and then transferred them to their experimental diets. We fed worms in each individual glass jar approximately 0.1g of dry powdered food per week, an amount meant to prevent limitation by food quantity (estimated to be ~0.012 g from Nehring et al. 2015). Worms in each individual well of the well plates were given 1 mL of food slurry after each sampling event (twice weekly until peak spore production and then once weekly after). The food slurry mixture was made by combining 1 g of powdered food with 1 L of dechlorinated tap water.

Glass jars (250 mL) with dechlorinated tap water and a substrate of washed play sand approximately 2 cm deep were placed in a chilled water bath to keep the worms at a constant 15 °C, a temperature known to be conducive to the production of spores (Blazer et al. 2003). Once worms produced spores in any of the replicates (i.e. individual jars) within a diet treatment, we transferred 6 of the worms from each jar of that treatment to individual wells of a 24 well plate, in order to measure spore production by individual worms. Each well plate row represented a single replicate, and replicates were distributed across well plates randomly using a random number generator. Well plates were also suspended in a chilled water bath in order to maintain constant temperature at 15 °C (see: Blazer et al. 2003).

We identified whether spores were being produced by any of the worms in a replicate by decanting water from each jar through 20 μm mesh and rinsing the contents of the filter into a 50

mL centrifuge tube with 15 mL of dechlorinated tap water. Spores were collected from individual wells of well plates by pipetting 200 µL from a well into a 96 well plate, then stained with saturated crystal violet at a ratio of 20 µL of crystal violet per 1 mL of sample. Stained samples were counted under 40X magnification using a Palmer counting cell. Jars were sampled weekly beginning 2 weeks after exposure to the *M. cerebralis* parasite. We sampled wells containing individual worms bi-weekly for 121 days post exposure (PE), then sampled once weekly until the end of the experiment at 174 d PE.

In total, 1600 individual worms were exposed to the parasite. Of those, 1280 were randomly distributed among the 80 experimental units (i.e., individual glass jars) in the study. 16 worms were placed in each individual glass jar, and each of the 5 diet treatments were replicated 16 times. Spore counts were averaged across the ~6 worms removed from jars and placed in individual well plates, yielding an average for each of the 80 experimental units. Worms that never produced spores were assumed to have not been successfully infected during exposure and were not included in average across individual worms or any further analyses of spore production.

Spore samples were collected until 177 d PE. At the conclusion of the experiment (177 d PE), worms were allowed to clear their guts for 24 hours and then were dried at 50°C, weighed, and analyzed for C, N, and P content. C and N content was determined by the UC Davis Stable Isotope Laboratory using a PDZ Europa ANCA-GSL elemental analyzer. Phosphorus content was determined by ashing, digesting with persulfate, and the ascorbic acid – molybdate spectrophotometric method (American Public Health Association 2005). Because all worms were randomly selected from the same source colony, mass at the end of the experiment was

considered analogous to growth and used instead of calculating a change in mass from a representative sample from the beginning of the experiment.

Statistical analyses were performed using SAS 9.7 (The SAS Institute 2013). Differences between diets were tested using generalized linear models and PROC GLIMMIX. Gamma distributions were used for spore data, and normal distributions for all other response variables. Normality was tested using the Shapiro-Wilke test. A bootstrap test of monotonicity (Murtaugh 2003) was applied to test for a TER in spore production over the range of diet stoichiometries. A quadratic regression was applied to spore production data if the test for monotonicity was significant.

Natural diet study

To assess how the patterns observed in the laboratory experiment compared with those in natural streams, we collected *T. tubifex* and fine benthic organic matter (FBOM) from 12 whirling disease positive sites in western Colorado, USA. Wild-caught worms were held in the laboratory at 15℃ and fed FBOM from their native streams. In contrast to the experimental diet study, spore production by individual worms was not quantified by transferring individuals to separate well plate cells. Thus, the number of spores produced per worm was calculated as an average based on the number of worms in each jar, which assumes all the worms are infected. Each whirling disease positive site was represented by a single experimental unit (jar). We chose not to sample individual worms for this phase of the study due to time constraints.

We determined whirling disease positive sites by collecting preliminary *T. tubifex* in July and August 2017, and analyzed worms for infection status and lineage type using qPCR, performed by Pisces Molecular LLC (Wade et al. 2004, Nehring et al. 2013). Infected worms for

the natural diet study were collected from positive sites in late October, which is the time that spore production occurs at these locations (Thompson et al. 2000, Nehring personal communication). Fifty worms from each site were randomly selected and placed in 250 mL glass jars with filtered water collected from the East River near the Rocky Mountain Biological Laboratory, and approximately 2 cm of washed play sand substrate. Also, in October, FBOM was collected from each site using a 0.09 m2 core tube; the core was placed into the substrate where the worms were found and the substrate stirred up with a garden spade. After 3 seconds had passed to allow sand and gravel to settle, 2 L Nalgene bottles were filled with the remaining slurry. FBOM was then dried in an oven at 60 ℃ and ground using a ball mill. In the laboratory, spore production was observed for 6 weeks.

Spores were collected by decanting the water from jars containing 50 worms from each site through 20 μm mesh and rinsing the contents of the filter into a 50 mL centrifuge tube with 15 mL of dechlorinated tap water. Spores were counted as described above for the experimental diet phase of the experiment. Samples were stained with saturated crystal violet at a ratio of 20 μL of crystal violet to 1 mL of sample. After staining, samples were counted at 40x magnification using a Palmer counting cell.

Statistical analyses were performed using SAS 9.7 (The SAS Institute 2013). We used a bootstrap test of monotonicity to test for a hump shaped relationship over the range of FBOM stoichiometries (Murtaugh 2003). If the bootstrap test was significant, a quadratic regression was applied, and the results of the experimental diet and natural diet were compared using ANCOVA.

RESULTS

Experimental diet effects on pathogen reproduction

Sixty-three of the 80 experimental units produced spores during the course of the experiment. On average, 82% of the individual worms in each of the 63 replicates produced spores. We assumed that worms that never produced spores were not successfully infected or died before producing spores. For unknown reasons, all worms fed the C:P = 134 diet died before spore production began, so that treatment was excluded (16 replicates). That diet has been used successfully to rear worms for spore production in the past; therefore, the 100% mortality observed in the C:P =134 treatment was not due to that diet's stoichiometry or other components of the diet. The additional experimental unit that was excluded was from the C:P = 35 treatment due to 100% mortality in that unit.

Pathogen reproduction, measured as the average rate of spore production during the 174 days post infection, ranged from 0.07 spores per worm per day to 2300 spores per worm per day. Pathogen production was significantly related to the natural log of worm diet C:P stoichiometry and that relationship was significantly hump shaped (Figure 1, Regression: n = 63, $F_{2,60} = 8.80$, $p < 0.01$, $R^2 = 0.23$. Bootstrap test of monotonicity: $p = 0.01$). The extreme value in the $\log_e$ (C:P) = 4.6 treatment did not influence the interpretation of the statistical analyses and we do not have any other reason to exclude it. Worms fed the diet with $\log_e$ (C:P) = 4.6 were significantly larger than the worms reared on other diets (Figure S1, ANOVA, $F_{3,59} = 10.95$, $p < 0.01$)

The average of the total number of spores a worm produced during the 174 d post exposure period ranged from 3860 to 45,538 total spores per worm. The total number of spores per worm during the 174 d post infection was significantly different across diet treatments (Figure 2, ANOVA: n = 63, $F_{3,59} = 10.91$, $p < 0.01$). We included data in figure 2 reported by

Nehring et al. (2015) to provide information on how many total spores *T. tubifex* exposed to 50 myxospores per worm and fed the C:P = 133.7 ($\log_e$ [C:P] = 4.9) diet would have been expected to produce. However, we note that differences in that study would be expected to cause reduced spore production relative to the conditions of this experiment.

T. tubifex Mortality and Duration of Spore release

Worms fed the lowest C:P diet experienced significantly higher mortality than worms fed other diets (Figure 3, ANOVA: n = 63, $F_{3,59} = 24.19$, $p < 0.01$). Average worm mortality ranged from 0 % in the highest C:P diet to 38 % in the lowest C:P diet.

The average number of days an infected worm produced spores before ceasing spore production or dying ranged from 45 days in the lowest C:P treatment to 87 days in the $\log_e$ (C:P) = 4.6 treatment. The two intermediate diet treatments resulted in significantly longer periods of spore production than the extreme diets (Figure 4, ANOVA: n = 63, $F_{3,59} = 20.46$, $p < 0.01$).

Field collected worms

Worms from 11 of the 12 whirling disease positive sites produced spores during the course of the experiment. The rate of spore production by infected worms collected in the field was significantly related to the natural log of diet C:P, but that relationship was linear and not hump shaped (Figure 5). In addition to a non-significant bootstrap test of monotonicity (Linear regression: n = 11, $F_{1,9} = 6.24$, $p = 0.03$, $R^2 = 0.41$. Bootstrap test for monotonicity: $p = 0.61$), no quadratic regressions resulted in a significant effect of the second order term. The mass of worms reared on natural diets of field-collected FBOM was not significantly related to the natural log of its C:P (linear regression, n = 11, $F_{1,9} = 0.02$, $p = 0.89$, $r^2 = 0.0024$).

DISCUSSION

We found that *Tubifex tubifex* diet C:P affected the production of infectious spores by its parasite *Myxobolus cerebralis*, for both worms fed experimental diets, and worms fed natural diets. Worms fed experimental diets demonstrated a hump-shaped relationship between diet C:P and spore production, with increasing diet C:P increasing spore production for low C:P diets, and decreasing spore production on high C:P diets; a difference of an order of magnitude was observed across treatments. Worms fed natural diets demonstrated a positive relationship between diet C:P and spore production over the entire range of diet stoichiometries. These data support the hypothesis that the parasite *M. cerebralis*'s spore production is related to the diet C:P stoichiometry of its host, *T. tubifex*, and expands the diversity of host-parasite pairs in which this relationship has been observed. Further, spore production by worms fed experimental diets supports the hypothesis that parasite reproduction is subject to a threshold elemental ratio in relation to host diet, a relationship that has not been previously observed. These results also suggest that the costs associated with resource imbalances may be passed on from hosts to parasites.

Our findings that changes in host diet C:P can affect parasite reproduction are consistent with previous work (Frost et al. 2008, Aalto et al. 2013, Narr et al. 2015). However, our results differ from these studies in important ways that help expand our understanding of the relationship between host diet stoichiometry and parasite reproduction: Frost et al. (2008) demonstrated host diet C:P effects on parasite reproduction over a comprehensive range of experimental diet stoichiometries, but did not find a hump shaped relationship like that presented here. This is possibly because the hosts in the Frost et al. (2008) study were reared on their experimental diets prior to exposure, confounding direct effects of host diet on parasite

reproduction via parasite nutrient acquisition, and indirect effects arising from diet mediated differences in host resistance to infection. This difference highlights interesting questions regarding the multiple ways that host diet may synergistically and antagonistically affect parasite reproduction. Aalto et al. (2013) showed that *Daphnia magna* infected with a microsporidian parasite had significantly higher spore loads when fed P sufficient (low C:P) diets compared to P limiting (high C:P) diets. Like in our study, Aalto et al. (2013) began all their hosts on a P sufficient diet and maintained them on that diet until after exposure to the parasite, suggesting that their results were due to direct effects on the parasite. However, binary P-limited and P-sufficient diets do not directly test the effects of diet stoichiometry, but rather host P limitation; by using a greater number of experimental treatments, we have demonstrated that direct effects of host diet stoichiometry on parasite reproduction exhibit a curvilinear relationship as opposed to a binary relationship with limitation. We also observed the opposite relationship in our field collected worms: highest spore production on high C:P diets, suggesting that P may be less important for myxozoan parasites than bacterial parasites. Finally, Narr et al. (2015) demonstrated that differences in host diet stoichiometry, even those from relatively recent changes, can affect parasite reproduction in field captured hosts. However, they showed an inverted hump shaped relationship, i.e. the hosts fed the intermediate C:P diet demonstrated the least pathogen reproduction; we can only speculate on the meaning of this difference. It is possible that Narr et al.'s (2015) results are not statistically different from a straight line and thus show increasing spore production with increasing diet C:P, similar to our results from field captured hosts. It is also possible that their results show an interaction between host and parasite TER curves, with the intermediate diet being near ideal for host, and poor for parasite while the low and high P diets confer advantage to neither.

The TER hypothesis has not previously been tested in a parasitic system to our knowledge. As parasites are consumers, they should theoretically demonstrate TERs. However, the unique characteristics of parasitic interactions compared to other type of consumer-resource interactions may influence the nature of parasitic TERs. Here we showed a TER in spore production over host diet stoichiometry. For most consumers, growth rate is the dependent variable relevant to TERs (Frost et al. 2006, Benstead et al. 2014), but for unicellular organisms growth rate may not be appropriate. This raises the question of what the most relevant dependent variable might be for multi-cellular macroparasites. Zehnder et al. (2009) observed that aphid *population* growth was highest on plants with intermediate foliar %N, which may or may not have been mirrored by individual growth rates, which were not measured. Additionally, consumer TERs are predicted to shift when food quantity is below a certain threshold (Halvorson et al. 2017), but because parasites are generally sessile and much smaller than their hosts, a specific parasite's TER may be less variable than that of other kinds of consumers (Sanders et al. 2018). Classically, the negative growth effects of excess nutrients have been attributed to the metabolic costs of processing those excess nutrients (Boersma et al. 2006), and there is evidence of this mechanism (Elser et al. 2016). Alternatively, we hypothesize that the reduced reproduction of the parasite on high P host diets in this study was due to excessive spore reproduction by the parasite and attendant host mortality due to high parasite burden, as opposed to the metabolic costs of processing excess P. However, more work is required to test this hypothesis. We are not the first to suggest that high availability of a nutrient may result in growth costs by mechanisms other than the metabolic costs of processing imbalanced diets. Monarch caterpillars (*Danaus plexippus*) feeding on the toxic leaves of *Asclepias curassavica* showed decreased growth in response to elevated foliar N, not because elevated N incurred metabolic

excretion costs, and not because elevated N was related to elevated toxin concentrations, but because the leaf toxin was either a more toxic compound, or because of some unknown post-ingestion interaction between the toxin and the excess N consumed (Tao et al. 2014).

Although we were only able to analyze data from 4 diet treatments, we are confident in our statistical analyses. First, the bootstrap test of monotonicity that we performed returned a probability of 1% that the relationship between spore production and $\log_e$(diet C:P) was monotonic. Second, we observed a very large range in spore production over $\log_e$(diet C:P). If addition of higher $\log_e$(diet C:P) treatments were to reveal a linear relationship, it would necessitate further increases in spore production of many orders of magnitude.

Our results also expand our understanding of the specific effects of host diet on spore production in the whirling disease system. Until now our understanding of the effects of diet on *M. cerebralis* spore production by infected *T. tubifex* was restricted to the differences among rearing them on sand, mud, and leaves amended with spirulina and lettuce. Infected worms reared on mud produced more spores and had longer infectious periods than worms reared on sand and leaves, suggesting that diet quality is possibly related to *M. cerebralis* spore production, as mud is rich in organic matter that would be available to the worms. Comparison with the Blazer et al. (2003) study is difficult because their results could have been due to substrate dependent stress the hosts experienced independent of food quality, or food quantity versus quality. The results from our field collected worms suggest that the effects of FBOM C:P stoichiometry on parasite reproduction are strong enough to overcome any effects due to variation in substrate structure across our study sites. Many previous whirling disease studies have been done using the experimental diet that we used as the basis for all of our experimental diet recipes (Nehring et al. 2015, Nehring et al. 2016). Our results help put this diet into context

for future researchers. The TER results indicate that in streams with FBOM of intermediate C:P that worms can have higher spore production than in streams containing FBOM with low or high C:P. Thus our understanding of and ability to predict *M. cerebralis* spore production, and hence, whirling disease risk to trout can be improved by knowledge of the stoichiometry of the worm host's potential food.

Interactions between host diet and lethal and non-lethal stressors may affect parasite performance and the TER. Our results suggest that lower spore production from hosts given diets with C:P lower than the TER may have been due to elevated stress and mortality on those diets. It is possible that those diets were nutritionally favorable to the parasite, but the individual worms with the highest parasite burdens died before they could finish producing spores, e.g. Brassard et al. (2009) who found that mortality increased exponentially with fluke burden in brook trout. Worms fed the lowest C:P diet had higher mortality, but we cannot say conclusively if this was related to parasite burden, highlighting an avenue for future inquiry.

The worms collected in Colorado and reared on natural diets of fine benthic particles from their natal streams produced substantially fewer spores per worm than those reared on experimental diets. One reason is that all of the worms used to calculate spore production per worm in the experimental diet study were *known* to be infected, but in the natural diet study we *assumed* that all worms were infected, which was probably not valid. Previous studies have found 1% to 8% *M. cerebralis* infection rates for wild caught *T. tubifex* (Zendt et al. 2000, DuBey et al. 2004, Lodh et al. 2011, Nehring et al. 2013). If natural prevalence rates are assumed to be 1%, however, the rate of spore production in wild caught worms is still 1-2 orders of magnitude lower than what would be expected from the analogous experimental diet. However, that range of rates does overlap with natural rates of spore production observed in previous

studies (Nehring et al. 2003, Nehring et al. 2013). It is possible that differences in the infection dosage, or number of myxospores the worms had access to, influenced spore production. However, previous work indicates that myxospore dose does not strongly affect spore production (Stevens et al. 2001). Being composed primarily of commercially produced fish foods and highly processed ingredients, the experimental diets were likely more labile than the natural diets, and thus the lower metabolic costs of digestion and the higher assimilation efficiency may have meant that a substantially greater fraction of ingested nutrients was passed along to the parasite. Finally, the wild caught worms at each site would have been feeding on different diets when they were exposed to the parasite, which may have influenced the likelihood that an ingested spore was able to successfully establish in the host (i.e. susceptibility/resistance).

There was no evidence of a hump shaped relationship in the natural diet data, however spore production on the natural diets showed the same trend as those in the experimental diet over the diet C:P values that they shared. For both the experimental and natural diets, increases in diet C:P resulted in increases in spore production for C:P diets less than 245. However, worms reared on experimental diets with a higher C:P than those measured in the streams had reduced spore production. Because we were able to build a significant statistical model relating host diet stoichiometry to parasite production in both phases of the study (experimental and natural diets), it should be possible to produce an SIR type epidemiological model for whirling disease that incorporates resource stoichiometry (sensu Vannatta et al. 2018). Incorporating resource stoichiometry into epidemiological models, for example by linking transmission, β, to host diet stoichiometry via spore production using our results, would result in more accurate and locally specific model predictions. This model could also help answer the question of whether there are nutrient thresholds that trigger or prevent epidemics (Bernot et al. 2018). Additional

opportunities to incorporate stoichiometry into disease models will arise as our understanding of the relationships between resource stoichiometry and various aspects of infectious disease dynamics becomes more refined and are investigated for more host-parasite pairings.

Because we were able relate host diet stoichiometry to parasite production using both experimental and natural diets, it should be possible to develop a SIR-type epidemiological model for whirling disease that incorporates resource stoichiometry (sensu Vannatta et al. 2018). Incorporating resource stoichiometry into epidemiological models, for example by linking transmission, β, to host diet stoichiometry via spore production using our results, would result in more accurate and locally specific model predictions. This model could also help answer the question of whether there are nutrient thresholds that trigger or prevent epidemics (Bernot et al. 2018). Additional opportunities to incorporate stoichiometry into disease models will arise as our understanding of the relationships between resource stoichiometry and various aspects of infectious disease dynamics becomes more refined and are investigated for more host-parasite pairings.

This work raises fundamental questions regarding the interplay between the effects of host diet stoichiometry and lability on parasite reproduction and on host resistance and tolerance. Future studies should investigate how host diet stoichiometry may influence both parasite reproduction and host resistance in antagonistic or synergistic ways. Similarly, do host and parasite TERs differ or overlap? and can the degree of overlap be useful in predicting how changes in resource stoichiometry will influence parasite population dynamics (Sanders et al. 2018)? Narr et al. (2015) showed that trematode infected snails produced the fewest cercaria on a diet with intermediate C:P, which may have been indicative of the host's TER. Frost et al. (2008) showed a relationship between host diet stoichiometry and parasite burden that was more

complex than the hump shape indicative of a TER, which may hint at the combined effects of host diet stoichiometry on host resistance and parasite nutrient limitation/acquisition. Overall, the results of this study demonstrate that the stoichiometry of host diet has a strong influence on parasite production, but the relationship is complex and sometimes nonlinear. Finally, we have shown that there are TERs in host diet stoichiometry where parasites achieve maximum reproduction.

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Table 2.1: Experimental diet stoichiometries and the percentages of each component by mass.

C:P ($\log_e$[C:P])	% Agar	% Spirulina	% Tetramin	% Algamac	% Bone meal
1464.1 (7.3)	85	13	0	2	0
487.7 (6.2)	70	25	2.5	2.5	0
133.7 (4.9)	0	60	30	10	0
94.5 (4.6)	20	35	30	10	5
34.6 (3.5)	0	10	20	10	60

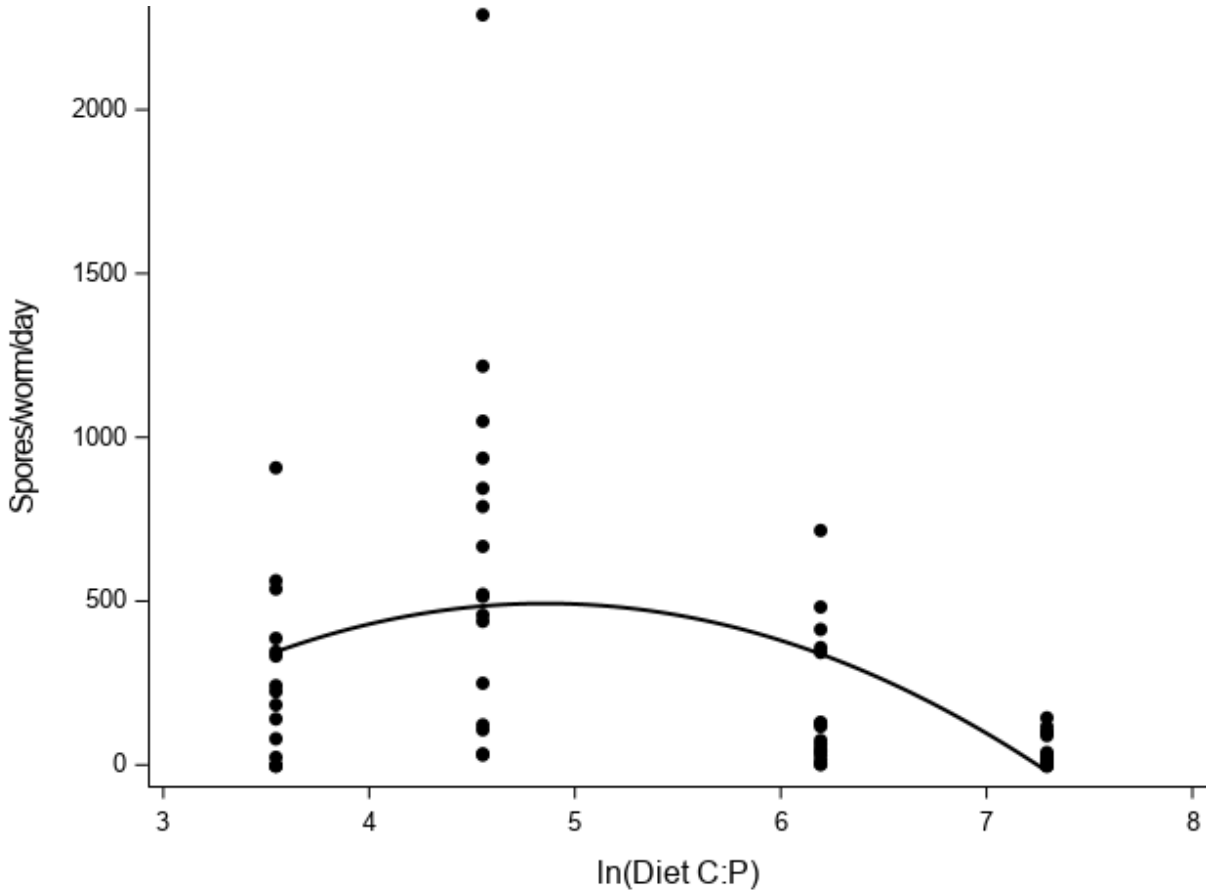

Figure 2.1: Pathogen spore production was significantly related to the natural log of worm diet C:P, and the relationship was significantly hump shaped, suggesting a TER. Trendline represents a significant quadratic regression. The extreme value in the $\log_e$ (C:P) = 4.6 treatment did not influence the interpretation of the statistical analyses and we do not have any other reason to exclude it.

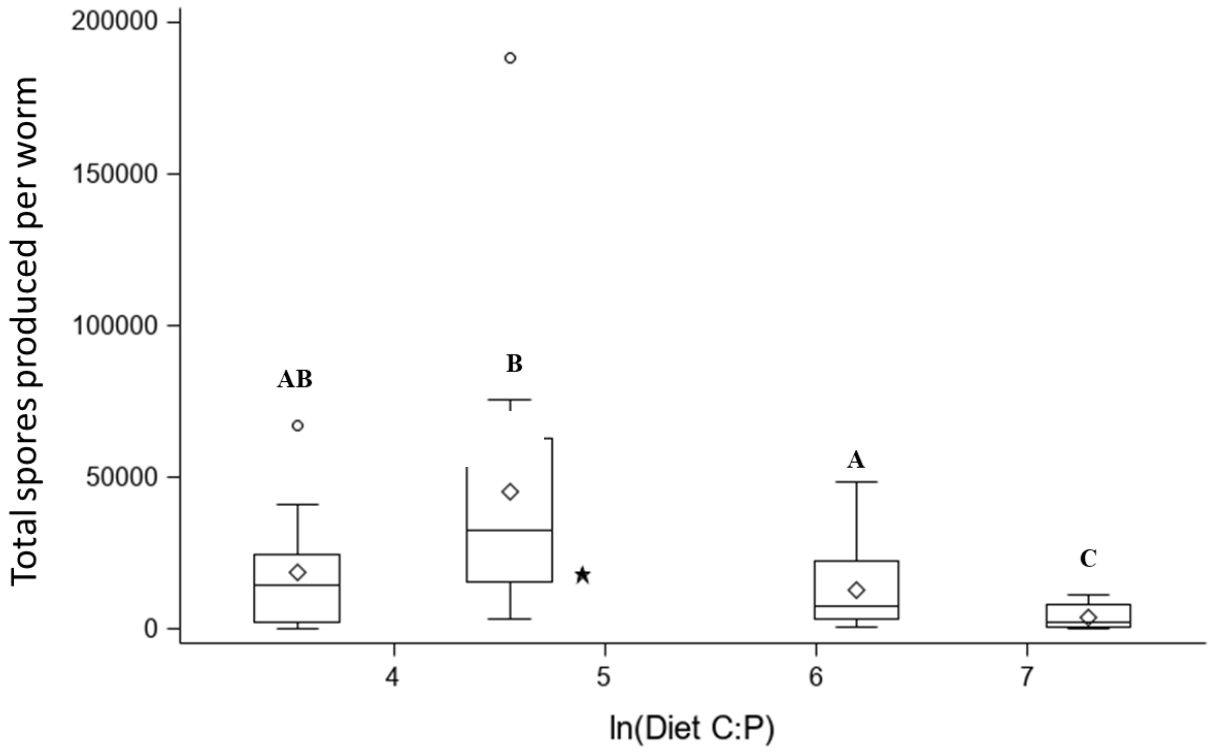

Figure 2.2: The total number of spores a worm produced during its infectious period was significantly different across diet treatments. Different letters represent significant differences as determined through Tukey-Kramer least square means comparisons. Star represents total spores produced as reported by Nehring et al. (2015). Box represents inter-quartile range, line in box represents median, bars represent maximum and minimum values (without outliers), open circles represent outliers (calculated as >1.5*IQR), and open diamond represents the mean.

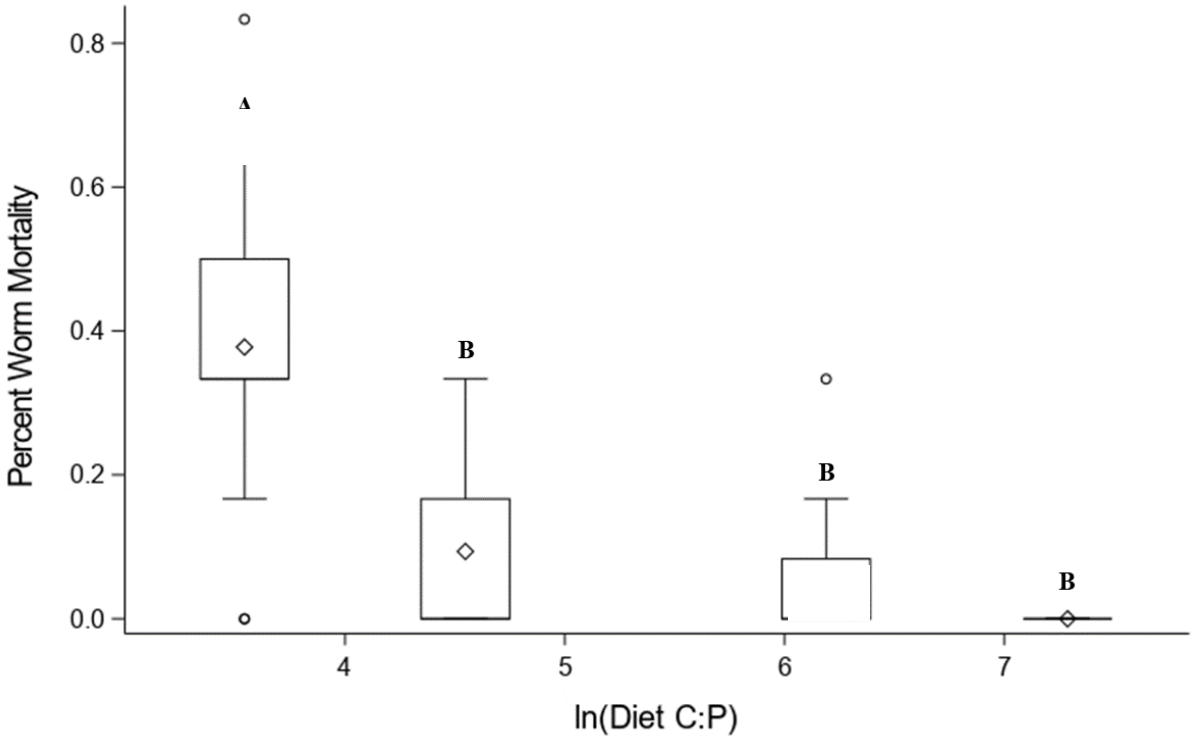

Figure 2.3: Mortality rates were significantly higher in the lowest C:P treatment than the other treatments. Different letters represent significant differences as determined through Tukey-Kramer multiple comparisons. Box represents inter-quartile range, line in box represents median, bars represent maximum and minimum values (without outliers), open circles represent outliers (calculated as >1.5*IQR), and open diamond represents the mean.

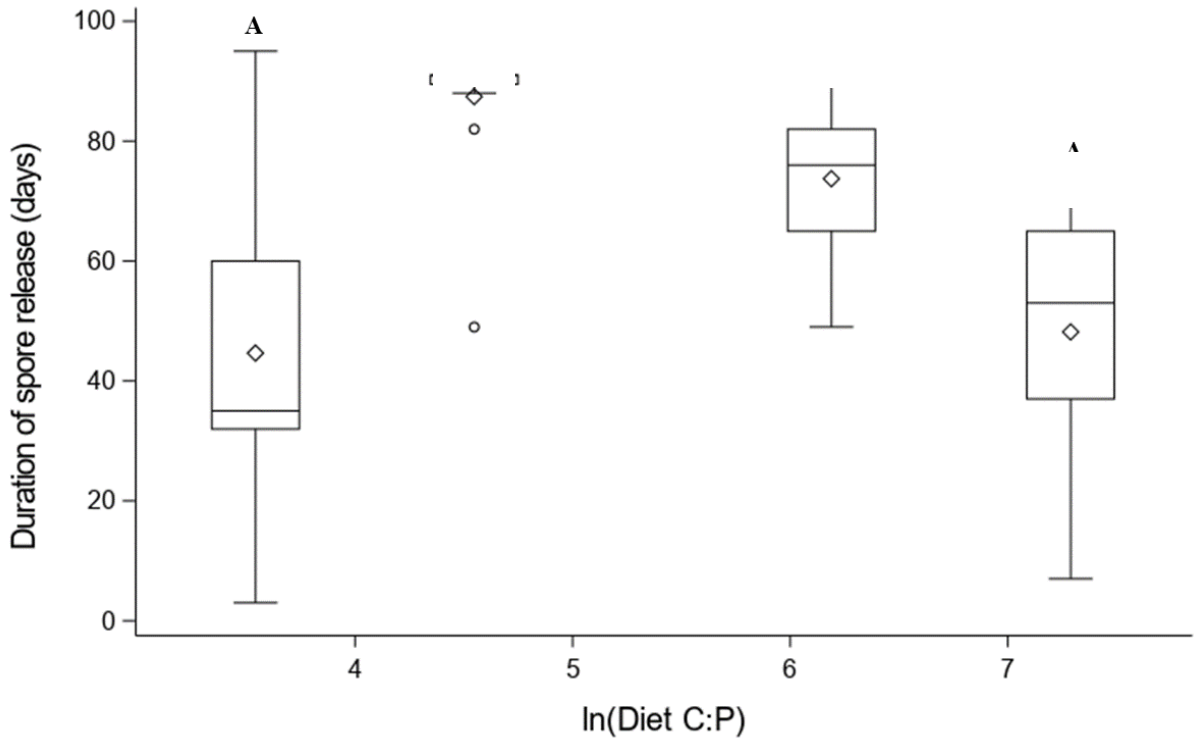

Figure 2.4: Duration of spore production was significantly different across diet treatments. Different letters represent significant differences as determined through Tukey-Kramer multiple comparisons. Box represents inter-quartile range, line in box represents median, bars represent maximum and minimum values (without outliers), open circles represent outliers (calculated as >1.5*IQR), and open diamond represents the mean.

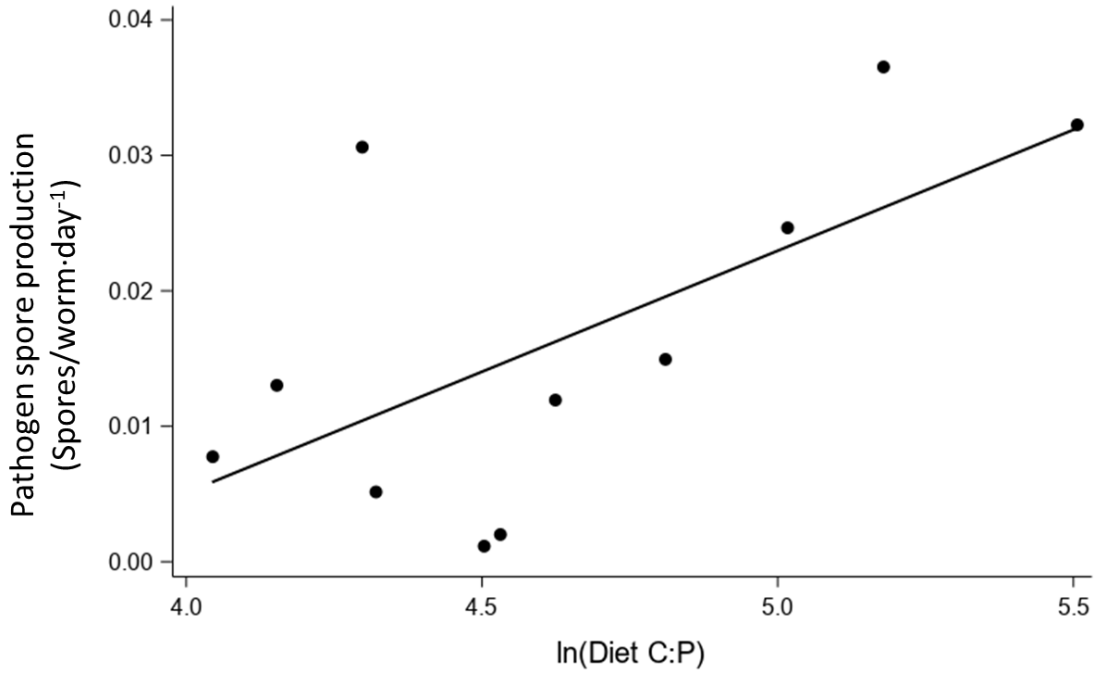

Figure 2.5: Figure 5. Worms collected in the field in Western Colorado showed a linear relationship between the natural log FBOM C:P and rate of spore production. Trendline represents significant linear regression.

CHAPTER 3: High Variability in the Effects of Parasites on Nutrient Recycling by their Hosts

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KEYWORDS: Stoichiometry, parasitism, CDNR, biogeochemistry

ABSTRACT

Consumer driven nutrient recycling provides an important flux of inorganic nutrients to primary producers. Parasites are consumers themselves, and can alter nutrient recycling by their consumer hosts. Previous work on the effects of parasites on host nutrient recycling suggest that parasitized hosts excrete nitrogen at greater rates, and that their excretion stoichiometry has a higher N:P than uninfected hosts. We tested the hypothesis that these are general traits of parasitized consumers by measuring the effects of parasites on their host's excretion rates in five pairs of insect host-parasite interactions (heptageniid-mermithid, baetid-mermithid, heptageniid-dipteran, perlodid-hydrachnidian, and apid-trypanosome).

Parasite effects on host excretion rates were highly variable and specific to individual host-pathogen pairs. Only the perlodid stonefly and one of the mayflies showed a parasite-induced change in excretion stoichiometry. The two mayflies parasitized by the mermithid showed parasite-induced changes in N and P excretion rates, but in opposite directions. The

dipteran parasite showed no effect on host excretion rates. Trypanosome infected bees demonstrated reduced N and P release, but the effect was not strong enough to produce a signal in release N:P stoichiometry. Stoichiometric mismatch between host and parasite did not predict parasite effects on host excretion. We show that parasite effects on host excretion rates and stoichiometry are highly variable and specific to individual host-pathogen interactions, and speculate that changes in host metabolism in response to parasitism, and non-linear parasite development may complicate the relationship between host and parasite stoichiometry and consumer driven nutrient recycling.

INTRODUCTION

The exchange of matter between consumers and their environment is an important control on the distribution of matter within ecosystems (Vanni 2002). Consumers also control the forms of available matter, notably by the processing of organic matter and excretion of inorganic nutrients, which are necessary for primary production (Vanni et al. 2006). Individual species may be keystone nutrient recyclers, meaning alterations in their abundance or distribution can have an outsized effect on nutrients in the ecosystem compared to their relative biomass (Small et al. 2011). Further, changes in consumer community structure can also affect productivity via associated changes in consumer driven nutrient recycling (CDNR). For example, shifts in the average body size of consumers can shift limitation from one nutrient to another (Elser et al. 1988). Changes in consumer diet, behavior and physiology, for example those related to predator exposure, can also translate to changes in nutrient cycling via CDNR (Olsen et al. 1986; Hawlena & Schmitz 2010; Dalton & Flecker 2014; Moody et al. 2015). Thus, predators have the potential to alter CDNR through consumptive effects (e.g. preferentially feeding on smaller

bodied prey) and non-consumptive effects (e.g. higher prey respiration rates and predator avoidance behaviors).

Parasites, like predators, can have profound consumptive and non-consumptive effects on their hosts' behavior and physiology that could influence nutrient recycling and stoichiometry (Moore 1995). Some of the myriad effects of parasites on their hosts' physiology include: castration (Codreanu 1935; Vance & Peckarsky 1996), increasing or decreasing activity (Poulin 1994), altering the production of pigments (Oetinger & Nickol 1982), decreasing feeding rate (Kyriazakis et al. 1998), and altering diet preference (Fielding et al. 2003). These types of parasite-induced changes to the host's diet and allocation of resources are likely to impact the mass balance of hosts and their rates and ratios of nutrient release. Additionally, parasites are themselves consumers and have their own tissues and metabolism, and therefore a different mass balance of nutrients than their host. Thus, we expect that parasites influence their host's role in nutrient recycling. Further, parasites can represent a substantial portion of community biomass (Kuris et al. 2008) especially if the parasitized host is considered an extension of the parasite phenotype (Preston et al. 2013), meaning parasite effects on CDNR can have substantial impacts on ecosystem nutrient cycling.

Parasites are generally smaller and faster growing than their hosts. Accordingly, the Growth Rate Hypothesis (GRH) predicts that parasites should have higher phosphorus (P) demand than their host's soft tissue (Elser et al. 2003). Therefore, all else being equal, consumers are expected to excrete inorganic phosphorus at lower rates and excrete higher N:P, as a greater proportion of their mass consists of parasite tissue. The results of previous work on snails infected with a trematodes, *Daphnia* infected with a bacterial parasite, and *Daphnia* infected with a fungal parasite suggest that infected hosts generally have higher mass-specific N excretion

rates than uninfected hosts, and stoichiometrically, excrete at higher N:P ratios (Bernot 2013; Narr & Frost 2016). They also suggest that parasite effects on P excretion may be complex and context dependent, despite the seemingly straightforward GRH based hypothesis that predicts parasites have high P demand compared to their hosts. These findings raise the question of whether higher N excretion rates and higher excretion N:P stoichiometry are general patterns across host-parasite relationships. They also raise the question of how informative tissue stoichiometry is in predicting the effects of parasites on host excretion.

The goal of this study was to test the hypotheses that parasitized hosts generally excrete more N, that infected hosts have higher N:P excretion stoichiometry than uninfected hosts, and that measurements of tissue stoichiometry can predict parasite effects on host excretion rates (e.g. if parasitized hosts have higher tissue N:P than unparasitized hosts, or if parasites have higher tissue N:P than unparasitized hosts, then does tissue stoichiometry reliably predict that parasitized host will have lower excretion N:P?)

METHODS

Host-parasite systems

We performed a series of independent studies comparing excretion rates and stoichiometries between infected and uninfected hosts from a variety of host and parasite pairs. The parasites studied include both endo- and ecto-parasites: nematodes, a mite, an insect, and a trypanosome. Hosts studied are all insects, primarily aquatic, but also a bee. We acknowledge that this range of host-parasite pairs is limited, but nonetheless, it still broadens our knowledge on the effects of host-parasite interactions on excretion.

Epeorus longimanus (Ephemeroptera: Heptageniidae), *Baetis bicaudatus* (Ephemeroptera: Baetidae), and *Rhithrogena hageni* (Ephemeroptera: Heptageniidae) are all grazing mayflies found in swift, high elevation streams in the American West (Dodds 1923). In the streams around the Rocky Mountain Biological Laboratory (RMBL), *B. bicaudatus* is the numerically dominant invertebrate, with densities well over an order of magnitude higher than the other most common taxa (Allan 1982). *Megarcys signata* (Plecoptera: Perlodidae) is a large stonefly also found in the same streams. Young nymphs feed on diatoms, filamentous algae, and detritus, but quickly shift toward predation on other insects as they get older (Cather & Gaufin 1975). *Bombus impatiens* (Hymenoptera: Apidae), the Common Eastern Bumble Bee, is found throughout Eastern North America. It is also a commercially important species used for crop pollination.

The mayflies *Epeorus longimanus* and *Baetis bicaudatus* are hosts to an endoparasitic mermithid nematode, *Gasteromermis spp*. (Nematoda: Mermithidae). An infected mayfly will host a single worm, which grows to fill the abdominal cavity of the host (Vance & Peckarsky 1996). Mermithid nematodes typically sterilize their hosts (Vance & Peckarsky 1996) and feminize male hosts (Vance 1996). It is unknown if the species of *Gasteromermis* that infects *B. bicaudatus* is the same species that infects other aquatic insects, as mermithid host specificity may be restricted to a single species, genus or family (Hominick & Welch 1980).

The heptageniid mayfly *Rhithrogena hageni* hosts the chironomid obligate parasite, Symbiocladius rhithrogenae (Diptera: Chironomidae; Codreanu 1930). The ectoparasitic chironomid attaches itself to the host mayfly under the wing pads, embeds its head in the soft tissues there and subsists by feeding on the host's hemolymph (Claassen 1922). Symbiocladius rhithrogenae sterilizes female hosts and early instar male hosts, but does not damage the testes of

later instar males enough to impact spermatogenesis (Soldan 1979). The host's fat bodies are exhausted by the parasite and it dies within a few days of the adult chironomid's emergence (Codreanu 1935).

The ectoparasitic water mite *Hydrachnidia sp.* (Arachnida: Trombidiformes) are a common parasite of the perlodid stonefly *M. signata*. The bright red mites are usually found attached under the wing pads or at the joint where the coxa meets the pleuron (personal observation). *Hydrachnidia spp.* parasitize a broad range of aquatic insects, including Plecoptera, Odonata, Diptera, Trichoptera, Coleoptera, and Hemiptera (Smith & Oliver 1986). The parasitic mites attach themselves to the host, penetrate through the cuticle with a blade-like mouthpart and feed on hemolymph and liquefied tissue (Smith 1988). The pathological effects of mite infestation are unknown in *M. signata*, but studies from other host species indicate that mite infestation can reduce fecundity, slow development, impair flight ability, and cause death (Smith 1988).

Finally, the trypanosome *Crithidia bombi* (Trypanisomatida: Trypanisomatidae) is a widespread chronic gut pathogen of bumble bees, including *Bombus impatiens* (Erler et al. 2012). It is horizontally transmitted via contact between individuals or from shared stores of food within nests (Erler et al. 2012), and has been showed to impair ovary development that can reduce colony size (Shykoff & Schmidhempel 1991).

Excretion measurements

To test the hypotheses that parasitized consumers excrete greater N:P and that differences between unparasitized host tissue stoichiometry and parasitized host and parasite tissue stoichiometry can predict the effects of parasitism on host excretion rates, we performed field

excretion trials on *E. longimanus*, *B. bicaudatus*, *R. hageni* and *M. signata*. Invertebrates were collected using a D-frame net with 500 µm mesh. *Epeorus longimanus* and *R. hageni* were collected from the East River near Gothic, CO. *Baetis bicaudatus* were collected from Upper Rock Creek and Marmot Creek in the Upper East River Valley. We selected these sites for *B. bicaudatus* because Upper Rock Creek has a low prevalence of parasitized *B. bicaudatus* and Marmot Creek has a high prevalence (B. Peckarsky and K. Cromwell, personal observation). Streamside excretion measurements were performed for *E. longimanus*, *R. hageni*, and *M. signata*, because infections could be reliably diagnosed in the field. For *E. longimanus* and *R. hageni*, each individual was held in a 200 mL Whirl-Pak bag with 100 mL of filtered (<1 µm Pall AE glass fiber filters) stream water for 1.5 to 2 hr. *M. signata* were held 1 or 3 to a bag in a 710 mL plastic Whirl-Pak bag with 120 mL of filtered stream water.

B. bicaudatus, and their hosted *Gasteromermis*, were too small to reliably diagnose as parasitized in the field, and so we collected individuals from both high and low prevalence sites, held them in mesocosms, and diagnosed them via dissection after collecting excretion samples. Mesocosms shared water from an unnamed tributary of the East River flowing off the east face of Gothic Mountain. Each mesocosm contained periphyton covered stones from the East River, which the *Baetis* were allowed to feed on for 3 days in order to minimize potential effects of diet differences between the high and low prevalence source streams. Because *Baetis* were too small to produce measurable excretion individually, ten individuals were placed together into each of 40 flow-through mesocosms. Thirty-three mesocosms were assigned a pre-determined mix of *Baetis* from the two locations to ensure a gradient ranging from low prevalence (all Upper Rock Creek) to high prevalence (all Marmot Creek) of *Gasteromermis*, by tenths, and each mix was replicated 3 times. The mix of *Baetis* in the remaining 7 mesocosms were determined using a

random number generator, which resulted in an infection prevalence of 0, 11, 20, 20, 37.5, 40, & 60 % in those mesocosms. After holding the *Baetis* in the mesocosms for 3 days, we performed excretion trials on the *Baetis* from each mesocosm as described above for *E. longimanus* and *R. hageni.*

Stream insects held in plastic bags containing 100 mL filtered (<1 µm Pall AE glass fiber filters) stream water were kept in a cooler with stream water. The temperature inside the cooler was recorded each time the lid was opened. Controls were setup by briefly dipping insects in a bag with 100 mL filtered stream water, after which control bags were treated the same as bags containing insects. Three parasitized controls and three unparasitized controls were collected each day we performed excretion trials.

Two *B. impatiens* colonies were provided by BioBest LTD, and were confirmed to be pathogen-free by screening the feces of 5 workers per colony. We infected one colony with *C. bombi* by haphazardly feeding the workers *Crithidia* inoculum once per week for 5 consecutive weeks. The *Crithidia* inoculum was created following the protocol of Richardson et al. (2015): Infected workers were dissected and their intestinal tracts (excluding the honey crop) were finely ground and centrifuged with distilled water. After resting at room temperature for 4-5 hours, *Crithidia* cells were counted with a Neubauer hemocytometer and diluted to a concentration of 1200 cells per µL with distilled water. The inoculum was then mixed 1:1 with 50% sucrose solution, yielding an inoculum with 600 cells per µL and 25% sucrose. Both colonies were supplied with food made of 30% sucrose solution mixed with ground honey-bee collected wildflower pollen and 30% sucrose solution nectar substitute ad libitum.

Thirty bees were removed from each colony into individual 5 dram (18.5 mL) vials that were placed at an angle permitting bee feces to collect in the bottom. Bees were held in the vials

on the lab bench at ambient room temperature (~20 °C), with the lights on and with ambient sunlight from open windows, for one hour. Fifteen vials containing bee feces were selected from each colony. Bumble bees excrete and egest simultaneously by releasing a wet fecal pellet. Separating the excreta and egesta was not possible, so we analyzed the total waste produced by the bees.

N and P excretion sample analyses

Samples from stream insect excretion measurements were collected in 30 mL borosilicate glass test tubes for soluble reactive P (SRP) analysis, and 60 mL plastic amber bottles for dissolved N analysis. Samples were analyzed the same day as collection for NH_4^+ and PO_4^- concentration. We used the ascorbic acid molybdate-blue method to measure SRP (American Public Health Association 2005), and the fluorometric method as modified by Taylor et al. (2007) to measure dissolved NH_4-N. The average concentration measured in the controls was subtracted from the concentrations measured in bags with insects. Samples with negative values after subtracting the controls were excluded. We calculated mass-specific excretion rates by dividing the rate of gross excretion by the host weight for unparasitized individuals or the combined weight of host and parasite for parasitized individuals.

Bombus impatiens fecal samples were digested using the simultaneous total N and total P digestion technique as described by Ebina et al. (1983). After digestion we measured dissolved P using the ascorbic acid molybdate-blue method (American Public Health Association 2005). We analyzed total N as nitrate using the second-derivative spectrophotometric method described by Crumpton et al. (1992).

Host and parasite mass and body stoichiometry

Stream insects were frozen in plastic bags until they could be dissected. *Hydrachnidia sp*. were counted during dissection. After thawing, parasites were removed from their hosts, and host and parasite were then independently placed into pre-weighed aluminum tins, then dried in an oven at 50°C. After drying for at least 24 hours, specimens were placed in a container with desiccant to prevent condensation while cooling. Once cooled to room temperature, the vials and tins were reweighed.

After weighing, we pooled parasitized hosts, unparasitized hosts, and parasites, ground them into a fine powder in glass vials, and weighed them for pooled tissue C and N analyses, which were performed using an elemental analyzer at the University of California Davis Stable Isotope Laboratory. We analyzed pooled tissue P by persulfate digestion and subsequent colorimetric analysis using the ascorbic acid molybdate-blue method.

Bees were sacrificed after we collected their fecal matter. Their intestinal tracts were dissected and centrifuged with 300 µL distilled water, and *Crithidia* counted using a 0.2 µL Neubauer hemocytometer. The radial cell length from each bee's right forewing was measured to predict bee mass (Harder 1982). *Bombus impatiens* tissue stoichiometry was not analyzed because of the destructive sampling required to count *Crythidia*.

Statistical analyses

All statistical analyses were performed in SAS 9.4 (The SAS Institute 2013). We tested for normality using the Shapiro-Wilk test. If data were normally distributed, then Levene's test was used to test for equal variance, and the Welch t-test was used when the assumption of equal variance was not met. If data were significantly non-normal, and outliers were present or group sample sizes were small (<15), then the exact Wilcoxon rank sum test was used. For

comparisons across more than 2 groups, we used ANOVA or exact Kruskal-Wallis. Tukey's HSD and Wilcoxon's test were used to perform multiple comparisons between groups. If data were non-normal, but had sufficient sample size and were free from outliers, the parametric test was used. Means and standard errors are reported for normally distributed data and medians and Interquartile ranges (IQRs) are reported for non-normally distributed data. Where leverage points and outliers were issues for ordinary least squares regression, robust regression by S-estimation was used (Rousseeuw & Yohai 1984).

RESULTS

Epeorus longimanus & Gasteromermis sp.

Of the 60 *Epeorus* spp. individuals that were collected, 41 were *E. longimanus* and 19 were *E. deceptivus*. None of the *E. deceptivus* were parasitized. Of the 41 *E. longimanus* individuals collected, 29 (71 %) were parasitized by *Gasteromermis* and 12 (29 %) were unparasitized. The dry mass of parasitized *E. longimanus*, which included the mass of *Gasteromermis*, was 43 % less than the dry mass of unparasitized individuals. (Figure S1. t-test, n=41 parasitized=29 unparasitized=12, $t_{39}=3.41$, $p_{2\ tailed} < 0.01$. The median dry mass of Gasteromermis was 0.49 mg (IQR=0.57) and constituted 30 % (IQR=15.44) of the parasitized host total dry weight. *Gasteromermis* mass increased significantly with increasing *E. longimanus* mass (linear regression, n=29, $F_{1,27}=29.64$, $p<0.01$, $r^2=0.52$).

The C:N:P of parasitized *E. longimanus* tissue was 214:39:1 (48.6 %C, 9.4 %N, 0.7 %P). The C:N:P of unparasitized *E. longimanus* tissue (not including *Gasteromermis*) was178:30:1 (48.6 %C, 10.2 %N, 0.6 %P). *Gasteromermis* tissue C:N:P was 384:49:1 (58.1 %C, 8.7 %N, 0.4 %P).

Parasitized *E. longimanus* had 328 % higher mass-specific N excretion rates than unparasitized individuals (Figure 1A. Wilcoxon, n=36 parasitized=25 unparasitized=11, S=136, z=-2.30, p=0.02). Mass-specific P excretion rates of parasitized individuals were 76 % higher than mass-specific P excretion rates of unparasitized individuals (Figure 1B. Wilcoxon, n=36, parasitized=25, unparasitized=11, S=130, z=-2.51, p=0.011). Excretion stoichiometry, analyzed as atomic N:P was not significantly different between parasitized and unparasitized *E. longimanus* (Figure 1C. Wilcoxon, n=36, parasitized=25, unparasitized=11, S=190, z=-0.45, p=0.66).

Baetis bicaudatus & Gasteromermis sp.

Of the 400 total *Baetis bicaudatus* held in microcosms, 351 survived to the end of the experiment and were used to estimate parasite effects on excretion. Of those, 102 (29 %) were parasitized with *Gasteromermis sp.* when dissected. Prevalence of parasitism in the microcosms at the end of the experiment, among the surviving *B. bicaudatus*, ranged from 0 to 100 %, with a median 22 % (IQR=29). Prevalence of parasitism was not significantly related to the total mass of mayflies and worms in an experimental unit (regression, n=39, $F_{1,37}$=1.12, p=0.30, r^2=0.03). The dry mass of *Gasteromermis sp.* was not significantly related to the dry mass of parasitized *B. bicaudatus* (regression, n=33, $F_{1,31}$=1.35, p=0.25, r^2=0.04). Median dry mass of *Gasteromermis sp.* individuals (calculated as the total worm dry mass divided by the number of worms) was 0.06 mg (IQR=0.10). The median contribution of *Gasteromermis* to the total dry mass of parasitized *B. bicaudatus* was 6.1 % (IQR=6.8)

Tissue C:N:P of parasitized *B. bicaudatus* (not including *Gasteromermis* tissue) was 291:47:1 (48.9 %C, 10.2 %N, 0.3 %P), for unparasitized *B. bicaudatus*, 407:73:1 (50.1 %C, 9.5 %N, 0.4 %P), and for *Gasteromermis* 193:27:1 (56.3 %C, 9.3 %N, 0.8%P).

Mass-specific N excretion rate significantly decreased with increasing *Gasteromermis* infection rate for *B. bicaudatus* (Figure 2A; robust regression, n=39, X^2=7.03, p=0.01, r^2=0.09). Mass-specific P excretion rate was also negatively correlated with increasing infection rate (Figure 2B; robust regression, n=38, X^2=5.28, p=0.02, r^2=0.09). Excretion N:P stoichiometry increased significantly with infection rate (Figure 2C; robust regression, n=38, X2 = 8.04, p= <0.01, r^2=0.18)

Rhithrogena hageni & Symbiocladius rhithrogenae

Forty-nine of the fifty-five mayflies we collected for this portion of the study were *Rhithrogena hageni*. Of the 49 *R. hageni* collected, 27 were parasitized by Symbiocladius rhithrogenae and 22 were unparasitized. Mean dry mass of parasitized *R. hageni* (including the mass of *S. rhithrogenae*) was 69 % that of unparasitized individuals. (Figure S2. t-test, n=48 parasitized=26 unparasitized=22, t_{46}=3.34, $p_{2\ tailed}$<0.01). The median dry mass of *S. rhithrogenae* was 0.41 (IQR=0.34) mg. The median percentage of total host mass contributed by *S. rhithrogena* ranged was 9.95 (IQR=14.5). *Symbiocladius* dry mass was not related to *R. hageni* dry mass (robust regression n=26, X^2=0.01, p=0.91, r^2=0.000).

The C:N:P of parasitized *R. hageni* tissue (not including *S. rhithrogenae*) was 294:51:1 (48.5 %C, 9.8 %N, 0.4%P). The C:N:P of unparasitized *R. hageni* tissue was 284:47:1 (47.1 %C, 9.1 %N, 0.4%P). Symbiocladius rhithrogenae tissue C:N:P was 264:37:1 (50.8%C, 8.3 %N, 0.5 %P).

Parasitized *R. hageni* did not have significantly different mass-specific N excretion rates than unparasitized individuals (Figure 6A. t-test, n=48 parasitized=26 unparasitized=22, t_{46}=0.41, p=0.69). Mass-specific P excretion rates of parasitized individuals were also not significantly different than that of unparasitized individuals (Figure 6B. Wilcoxon, n=43 parasitized=23 unparasitized=20, S=420, z=-0.48, p=0.64). Excretion N:P presented a non-statistically significant trend of higher excretion N:P by unparasitized *R. hageni* (Figure 6C. Wilcoxon, n=43 parasitized=23 unparasitized=20, S=517, z=-1.86, p=0.06).

Megarcys signata & Hydrachnidia sp.

Of the 163 individuals collected, 101 (62 %) were parasitized with mites, and 62 (38 %) were unparasitized. These were divided among 58 Parasitized experimental units (bags), and 34 unparasitized units. The median number of mites infecting a parasitized *M. signata* individual was 12 (IQR=14). The average dry mass of parasitized individuals (including *Hydrachnidia* mass) was 47 % greater than the average mass of unparasitized individuals (Figure S3. Welch's t-test, n=92, parasitized = 58 unparasitized=34, $t_{88.52}$=5.19, p<0.01). The median dry mass of an individual mite was 5.5 µg (IQR=5), and neither total mite dry mass, nor individual mite dry mass was significantly related to the dry mass of parasitized *M. signata* (individual mite mass, robust regression, n=58, X^2=2.7, p=0.10, r^2=0.03; total mite mass, robust regression, n=58, X^2=0.57, p=0.45, r^2=0.00) The median contribution of mites to the total dry mass of each experimental unit (bag) was 0.15 % (IQR=0.17). The number of mites in a parasitized experimental unit was not related to the dry mass of *M. signata* (robust regression, n=58, X^2=2.24, p=0.14, r^2=0.04).

The tissue C:N:P of parasitized *Megarcys signata* was 144:23:1 (49.7 %C, 9.3 %N, 0.9 %P), and the tissue C:N:P of unparasitized *M. signata* was 160:27:1 (50.3 %C, 9.9 %N, 0.8 %P). There was not enough dry mass of *Hydrachnidia* to analyze %C and %N, but mite tissue had 0.2 %P.

No statistically significant effects of *Hydrachnidia* infection on *M. signata* N excretion rates were observed (Figure 4A. Kruskall-Wallis, n= 48, high parasitized=13, low parasitized=16, unparasitized=19, df=2, X^2 =0.09, p=0.95). Mass-specific P excretion rates were 268 % higher for high parasite burden and unparasitized individuals compared to low parasite burden individuals (Figure 4B. Kruskal-Wallis, n=47, high parasitized=11, low parasitized=15, unparasitized=21, df=2, X^2 =12.27, p=0.022). These results were reflected in the N:P excretion stoichiometry, which was significantly lower for high burden and unparasitized individuals compared to low burden individuals (Figure 4C. Kruskall-Wallis, n=37, high parasitized=8, low parasitized=12, unparasitized=17, df=2, X^2 =8.40, p=0.015).

The tissue C:N:P of parasitized *Megarcys signata* was 144:23:1 (49.7 %C, 9.3 %N, 0.9 %P), and the tissue C:N:P of unparasitized *M. hydrachni* was 160:27:1 (50.3 %C, 9.9 %N, 0.8 %P). There was not enough dry mass of *Hydrachnidia* to analyze %C and %N, but mite tissue had 0.2 %P.

Bombus impatiens & Crithidia bombi

The mass of infected bees (0.35±0.06 g) was not significantly different from the mass of uninfected bees (0.38±0.03 g; t-test, n=30 parasitized=15 unparasitized=15, t_{28}=1.37, p=0.18). The median *Crithidia bombi* cell load for parasitized individuals was 106,500 cells

(IQR=139,500). *Crithidia bombi* cell load was positively related to bee mass (robust regression, n=15, X^2=16.28, p<0.001, r^2=0.49).

Mass-specific N release rate was 49 % less for infected bees compared to uninfected bees (Figure 5A. Wilcoxon, n=30, parasitized=15, unparasitized=15, S=181, z=2.12, p=0.03). Mass-specific P release was 38 % lower for infected bees compared to uninfected individuals, (Figure 5B. Wilcoxon, n=30, parasitized=15, unparasitized=15, S=177, z=2.28, p=0.02) The average fecal N:P of infected individuals was not significantly different from uninfected bees (Figure 5C. t-test, n=30, parasitized=15, unparasitized=15, t_{28}=0.64, p=0.53).

DISCUSSION

Overview

We investigated whether parasitized hosts generally excrete more N than unparasitized hosts, and have higher excretion N:P. Overall, only *E. longimanus* parasitized with *Gasteromermis sp.* demonstrated evidence that parasitized hosts excrete N at higher rates. *Baetis bicaudatus* parasitized with *Gasteromermis* demonstrated the opposite: they had lower mass-specific N excretion rates compared to unparasitized *B. bicaudatus*. Bees infected with *Crithidia bombi* also demonstrated lower mass-specific N recycling rates. Neither *Megarcys signata-Hydrachnidia* nor *Rhithrogena hageni-Symbiocladius rhithrogenae* showed any parasite effects on host N excretion rates (Table 3.1).

Compared to N, parasites had more consistent effects on P excretion rates. *Megarcys signata-Hydrachnidia*, *Baetis bicaudatus-Gasteromermis*, and *Bombus impatiens-Crithidia bombi* all demonstrated parasite induced reductions in P excretion rates. However, *Gasteromermis* increased rates of P excretion by parasitized *E. longimanus*. As with N,

Rhithrogena hageni-Symbiocladius rhithrogenae showed no parasite effects on any excretion rates (Table 3.1).

Only data from *M. signata* and *B. bicaudatus* presented statistically significant parasite effects on excretion N:P stoichiometry: both showed a parasite induced increase in excretion N:P. The *M. signata* results were dependent on the host's parasite load, similar to the result found for snails parasitized by a trematode (Mischler et al. 2016). *Rhithrogena hageni-S. rhithrogenae* data suggested a trend of reduced excretion N:P by parasitized individuals, but the trend was not statistically significant, and mass-specific rates of N and P excretion showed no relationship to infection status. *E. longimanus-Gasteromermis* and *B. impatiens-C. bombi* had parasite effects on both rates that precluded any significant change in their stoichiometric ratio. Our results lead us to reject the hypothesis that parasitized hosts are generally leakier in N, and excrete higher N:P, a result that has been seen across previous studies (Bernot 2013; Mischler et al. 2016; Narr & Frost 2016). Instead, in our analysis, parasites are more likely to influence P than N, and are more likely to reduce excretion rates by their hosts rather than increase them. However, when parasites did induce a significant change in excretion N:P in our study, it was an increase (Table 3.1).

Overall, our results demonstrate that parasite effects on host excretion rates and stoichiometry are variable and highly specific to individual host-parasite interactions. Even closely related parasites infecting relatively closely related hosts had dissimilar effects on excretion rates. For example, *Gasteromermis* spp. had opposite effects on rates of P and N excretion by the mayflies *Epeorus longimanus* and *Baetis bicaudatus*: parasitized *E. longimanus* excreted more of both N and P, while parasitized *B. bicaudatus* had lower mass-specific excretion rates.

Host and parasite tissue stoichiometry

The growth rate hypothesis (Elser et al. 1996) predicts that relatively small and fast growing organisms should be more P rich than larger and slower growing organisms, and thus have high P demand. Therefore we predicted that parasites would be more P rich, and have higher P demand than their hosts. Indeed smaller parasites are more P rich than larger parasites (Paseka & Grunberg 2018), and the tissue of parasitic trematodes has been shown to be more P rich than the tissues of their host snail (Chodkowski & Bernot 2017). In this study, two parasites had more P rich tissues than their hosts (*B. bicaudatus-Gasteromermis*, *R. hageni-S. rhithrogenae*), however two parasites had P deplete tissue compared to their host (*E. longimanus-Gasteromermis*, *M. signata-Hydrachnidia*). Notably, the difference between host and parasite P content was not consistent for *Gasteromermis*, nor the external parasites (*S. rhithrogenae* & *Hydrachnidia*). These results suggest that parasites are not more P rich than their hosts as a general rule, and that even closely related parasites or parasites with apparently similar traits cannot be presumed to have similar stoichiometric mismatches with their hosts. Relationships between unparasitized host tissue stoichiometry, parasite tissue stoichiometry, and parasitized host tissue stoichiometry might help explain interaction specific effects of parasites on host nutrient release rates. Bernot (2013) observed trematode induced shifts in snail excretion that were consistent with the hypothesis that high P parasite tissues were depleting host P and reducing host P release rates. However, elevated N release rates were not consistent with measurements of tissue N content. Narr and Frost (2015) found that bacterium induced changes in the excretion rates of *Daphnia* were only explained by considering both parasite induced changes in host tissue stoichiometry and parasite induced changes in host metabolism. In this study, differences between parasite tissue stoichiometry and unparasitized host tissue

stoichiometry were more often inconsistent with parasite related changes in excretion rates than they were consistent.

Gasteromermis parasitizing *E. longimanus* has both lower %N and %P than unparasitized hosts, and parasitized *E. longimanus* excreted greater N and P. This supports the hypothesis that stoichiometric mismatch between host and parasite drove differences in host excretion rates. Additionally, parasitized *E. longimanus* had lower %N and higher %P than unparasitized *E. longimanus*, suggesting that parasitism led to lower N assimilation and higher P assimilation.

Gasteromermis parasitizing *B. bicaudatus* reduced host excretion of both N and P, but had lower tissue %N and higher %P than their hosts. In this case, tissue %P agrees with excretion rates, but tissue %N does not. This could be explained by the elevated %N in parasitized individuals, and evidence of some metabolic or physiological change in the host. Parasitized *B. bicaudatus* had lower %P than unparasitized individuals; the difference was smaller than the difference between the parasite and unparasitized individuals, so it could have been hidden by the high P demand of *Gasteromermis*. *Gasteromermis* had a tissue %P 1.6 times that of unparasitized *B. bicaudatus*, but only represented 6.1 % of total parasitized host dry mass, so it's unlikely that *Gasteromermis*'s mismatch with the host in %P, and sequestration of host P in *Gasteromermis* tissues was directly driving its effect on P excretion.

Further, *Gasteromermis* is known to castrate *B. bicaudatus* (Vance & Peckarsky 1996), which would be expected to lead to increased P excretion as the P demands of castrated mayflies should be lower, sans the demand of P rich gonadal tissue. Reduced P excretion observed in parasitized *B. bicaudatus* indicates that even such a significant physiological change is not enough alone to predict the effects of parasitism on host excretion. Parasitized *B. bicaudatus* present numerous behavioral changes as well (Vance & Peckarsky 1997), and thus changes in

excretion are likely an emergent property of parasitism that integrates a number of other more direct effects of the parasite on the host.

One possible explanation for the different effects of *Gasteromermis* we observed between *E. longimanus* and *B. bicaudatus*, aside from the fact that they are likely different species of *Gasteromermis*, is the relative developmental stage of the parasite. *Gasteromermis* infecting *B. bicaudatus* were smaller relative to the host, than those *infecting E. longimanus*, and *B. bicaudatus* were sampled earlier in the summer. Mermithid worms infecting locusts (*Schistocerca gregaria forskal*) demonstrated a non-linear growth rate, with growth rate and protein synthesis highest during the final third of development (Gordon & Webster 1972). Thus, the observed effects of *Gasteromermis* on *B. bicaudatus* tissue stoichiometry and excretion rates may be indicative of the host's immune response, while the observed effects of *Gasteromermis* on *E. longimanus* tissue stoichiometry and excretion rates may be more indicative of the parasite's metabolism and pathogenic activity.

Mermithids infecting locusts meet their nitrogen demand during the time of highest growth by catabolizing host fat body and hemolymph proteins (Gordon et al. 1973). If the Mermithid is imperfect in assimilating those liberated amino acids they may be excreted by the host. Indeed, mermithids have been shown to preferentially take up some amino acids at much higher rates than others (Gordon & Webster 1972). Accordingly, the infected *E. longimanus* were depleted in N relative to uninfected individuals, and had higher N excretion rates. In comparison, parasitized *Baetis* were enriched in N relative to uninfected individuals, and demonstrated reduced N excretion rates. This could be attributed to the production of immunological proteins, or reduced consumption, a common host response to infection. Infected *Baetis* were also depleted in C relative to uninfected individuals, which could be indicative of

reduced consumption. These results are similar to those reported for predator exposed guppies, suggesting that they could be a metabolic response to parasitism independent of reduced consumption (Dalton et al. 2018).

Further, parasitized *E. longimanus* were enriched in P relative to uninfected individuals, while also showing elevated P excretion rates. Conversely, parasitized *Baetis* were depleted in P and demonstrated reduced P excretion rates. *Gasteromermis* infecting *E. longimanus* had lower tissue %P than *Gasteromermis* infected *Baetis*. Efficient reabsorption of aborted oocytes (Gordon et al. 1973), and low P demand by *Gasteromermis* (relative to N) may explain the relative increase in tissue %P in infected *E. longimanus* despite increased P excretion rates, while reduced consumption and high P demand by *Gasteromermi*s preparing for rapid growth may explain the reduced tissue P and excretion P in infected *B. bicaudatus*. It has been shown previously that in the final days of mermithid growth, protein incorporation slows relative to growth in total dry mass (Gordon & Webster 1972). Assuming that *Gasteromermis* infecting *E. longimanus* were further in their development than those infecting *B. bicaudatus*, our results agree with those observations: *Gasteromermis* infecting *E. longimanus* had higher tissue C:N ratios than those infecting *B. bicaudatus*, likely reflecting accumulation of energy stores in preparation for the worms' free living stage.

Symbiocladius rhithrogenae had lower %N and higher %P than unparasitized *R. hageni*, but had no statistically significant effect on *R. hageni* excretion rates. Parasitized *R. hageni* had slightly higher %N and slightly lower %P than unparasitized individuals, but the differences were much smaller than for other hosts. Of all the host-parasite pairs, *R. hageni* and *S. rhithrogenae* had the most similar tissue chemistry. *R. hageni* and *S. rhithrogenae* are the most closely related host-parasite pair, phylogenetically. We hypothesize that parasites that are more

closely related to their hosts, or that have had longer to co-evolve with their hosts, will have nutritional demands more similar to the tissues they parasitize and will have less effect on on host excretion. However, more data on more host-parasite interactions must be collected before this hypothesis can be tested.

Finally, *Hydrachnidia* had lower %P than *M. signata*, but at high levels of parasite burden had no effect on *M. signata* P excretion rate, and at low levels of parasite burden reduced *M. signata* P excretion rate. Parasitized *M. signata* had higher tissue %P than unparasitized *M. signata*. Similar to the results of *E. longimanus* and *B. bicaudatus*, comparing the effects of low burden *M. signata* to high burden *M. signata*, may reveal a deeper understanding of the interplay between potentially confounding effects of parasites on host excretion. Effects of mite parasitism at low levels may be more indicative of parasite induced changes in host metabolism, while the effects of high levels of parasitism may be more indicative of the parasite's own metabolism and consumptive/pathogenic effects on the host. Reduced P excretion at low levels of parasite burden could be indicative of reduced consumption, or a shift in the host toward more P conservative metabolism. The elevated tissue %P of parasitized individuals suggests the latter. Whereas the higher rates of total P excretion of high burden *M. siganata* relative to low burden insects may be the result of elevated P excretion by the low %P mites.

Thus, while our results fail to support the hypothesis that stoichiometric mismatch between host and parasite alone can explain the effects of parasites on CDNR. Tissue chemistry mismatch between parasite and unparasitized hosts seems to best explain excretion rates when parasite burdens were higher, and when host and parasite life history are taken into account. We hypothesize that as parasite burden (as percent mass of total parasitized host) increases, and mismatch between host and parasite increases, the metabolism and physiology of the parasite

increasingly drives the excretion rates and chemistry of the host. Our results for *Gasteromermis* in *E. longimanus* and *B. bicaudatus* add some depth and nuance to our GRH based prediction that parasites should be more P hungry than their hosts. Non-linear *Gasteromermis* growth means that early in the parasite's development, the host is growing faster, while at later stages the parasite is growing faster, yet the parasite possesses higher tissue %P than the host in the earlier stage of development and lower %P in the later stage. This could be because the parasite is enriching itself in P early, in order to support rapid growth later. Future studies should consider measuring diet stoichiometry and ingestion rates, similar to Narr and Frost (2015), as well as metabolomics, to gain a deeper, mechanistic understanding of how parasites affect host excretion.

Comparing parasites and predators

Our results are comparable to previous studies of parasite effects on host excretion. Although wild-caught snails naturally parasitized by trematodes demonstrated no effect of parasitism on excretion rates (Chodkowski & Bernot 2017), snails reared and parasitized in the lab showed 108 % higher mass-specific N excretion and 29 % higher mass-specific P excretion rates relative to unparasitized snails (Bernot 2013). *Daphnia* raised on an intermediate C:P diet and experimentally infected by the bacterium *Pasteuria ramosa* or the microsporidian *Hamiltosporidium tvarminnensis* showed 80 % and 50 % increases in mass-specific N excretion rates and 128 % and 77 % increases in mass-specific P excretion rates, respectively (as estimated using Data Theif III; Tummers, 2006; Narr & Frost 2016). In our study, low burden parasitized *M. signata* showed a 70 % decrease (equivalent to a 230 % increase) in P excretion compared to unparasitized individuals, and parasitized *B. bicaudatus* showed an estimated 64 % decrease

(equivalent to a 179 % increase). Parasitized *E. longimanus* showed a 76 % increase in mass-specific P excretion rate. Together, our results with those that have been previously reported indicated that parasites can have large impacts on host excretion rates.

The impacts of some of the parasites in our study on host excretion rates were much larger than published non-consumptive effects of predators on prey excretion rates. Grasshoppers (*Melanoplus femurrubrum*) exposed to the American nursery web spider (*Pisuarina mira*) released 40 % more N than unexposed grasshoppers (Hawlena & Schmitz 2010). In comparison, *Crithidia bombi* infected bumble bees in our study demonstrated a 49 % decrease in N release (equal to an increase of 97 %). Trinidadian guppies (*Poecilia reticulate*) exposed to chemical cues from the guppy predator *Crenicichla alta* had 17 % lower mass-specific N excretion rates relative to unexposed guppies (Dalton & Flecker 2014). In our study, parasitized *E. longimanus* had 328 % higher mass-specific N excretion rates (equal to a 77 % decrease), and parasitized *B. bicaudatus* had an estimated 33 % lower mass-specific N excretion rate than unparasitized individuals. Not all of the parasites in our study elicited changes in excretion rates in their hosts, but those that did elicited changes much stronger than those reported for predators.

Perhaps it is not surprising that parasites appear to have a stronger effect on host excretion rates than the presence of predators does on prey. Parasites have their own metabolism and consumptive effects on their host, in addition to their non-consumptive effects on host physiology. Comparing mechanisms across host-parasite pairs, as well as across predator-prey pairs and between parasites and predators is difficult. For example, decreased excretion rates in stressed prey and parasitized hosts could be due to metabolic responses to those respective stimuli, or due to reduced consumption. Additionally, reduced excretion rates in parasitized hosts could be due to the parasite shunting nutrients from the excretive pathway toward its own

growth. Investigators have begun to disentangle these effects, however. Reduced N excretion in predator stressed guppies has been shown to be caused by an immediate shift to N conservative metabolism, and not directly the result of reduced consumption rates (Dalton et al. 2018). Conversely, some prey exhibit increased N excretion in response to predation risk, and this appears to be related to stress induced catabolism (McDonald & Wood 2004; Hawlena & Schmitz 2010). Similarly to these opposing metabolic responses to predation risk, we observed divergent responses to parasitism that followed similar lines: elevated levels of N excretion in parasitized *E. longimanus* observed in our study are hypothesized to be due to catabolism of host tissue, while decreased P excretion in low burden parasitized *M. signata* are hypothesized to be due to a parasitism induced shift to P conservative metabolism.

Conclusion

Overall our results show that the effects of parasites on host excretion rates and excretion stoichiometry are highly specific to individual host and parasite interactions, and can differ substantially even between hosts of the same order parasitized by parasites of the same genus. Parasite effects on host excretion are also likely non-linear over the course of host and parasite ontogeny. However, reductions in host P excretion rates by parasites are probably common, especially in P limited systems similar to the streams in this study (Fuller & Peckarsky 2011). Our results suggest that differences between parasite tissue stoichiometry and unparasitized host stoichiometry alone do not necessarily predict the direction of parasite effects on host excretion rates. Changes in host metabolism in response to parasitism and host nutrient limitation likely complicate this relationship and lead to errant predictions at low levels of parasitism, as

evidenced by the differences observed between low and high infection *M. signata* and between infected and uninfected *B. bicaudatus*.

Parasites are widespread and can represent substantial portions of community biomass, exceeding that of top predators (Kuris et al. 2008), and as shown here can have effects on host excretion much stronger than the non-consumptive effects of predators on prey. Modeling suggests that parasites can substantially impact ecosystem level nutrient cycling (Mischler et al. 2016). Thus ecosystem ecology can greatly benefit from understanding the complex and interaction specific effects of parasites on ecosystem function (Preston et al. 2016).

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AUTHOR CONTRIBUTIONS

A.J. Sanders developed the ideas in this manuscript, designed and carried out the studies, analyzed the data, and drafted the manuscript for publication. B.W. Taylor assisted in designing the study and developing the ideas. K.J. Cromwell assisted in designing and carrying out the

Baetis bicaudatus study. J.J. Giacomini assisted in carrying out the *Bombus impatiens* study. All authors contributed to the editing of the manuscript.

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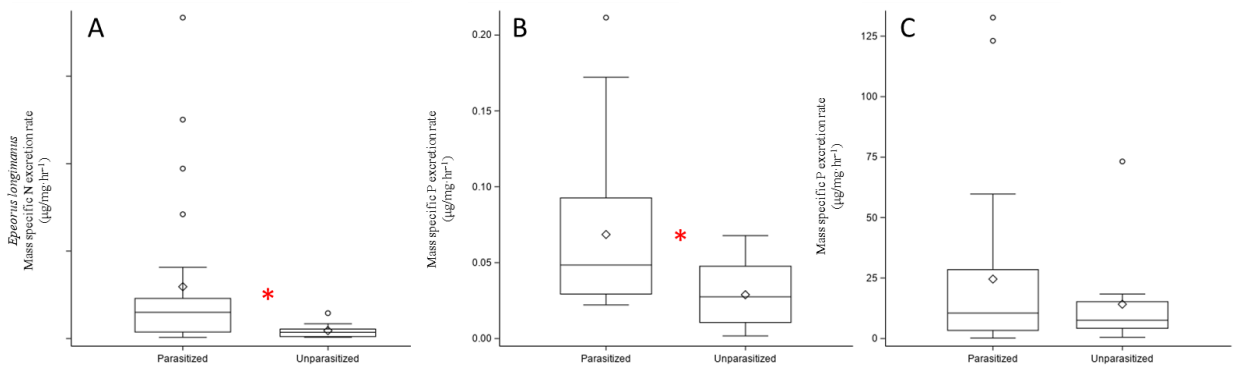

Figure 3.1: A) Parasitized *Epeorus longimanus* had significantly higher mass-specific N excretion rates the unparasitized individuals. B) Parasitized individuals also had significantly higher mass-specific P excretion rates. C) There were no significant differences in mass-specific N:P excretion. Boxes represent the interquartile range (IQR); whiskers represent the 1st and 4th quartiles; the middle horizontal line in each box represents the 2nd quartile, or median; the diamond in each box represents the mean, and circles represent outliers calculated as 1.5*IQR. Asterisks identify significant differences.

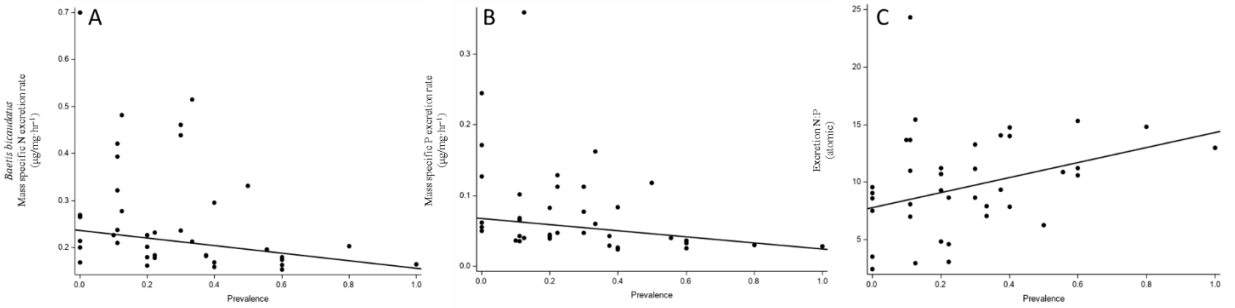

Figure 3.2: A) Increasing prevalence of *Gasteromermis sp.* infection was significantly related to decreasing mass-specific N excretion rates B) Increased prevalence was also related to decreasing mass-specific P excretion rates. C) The effects of the parasite on P excretion were not evident as a significant change in excretion N:P stoichiometry.

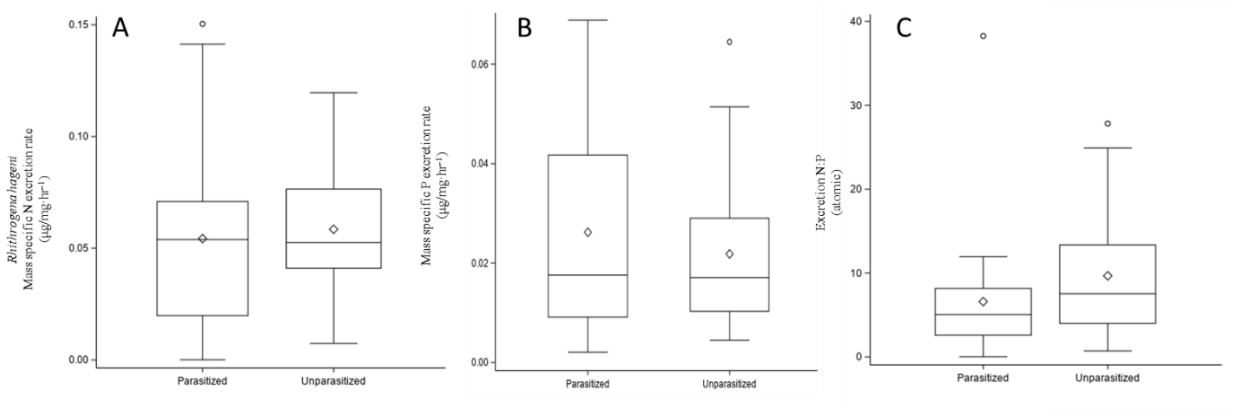

Figure 3.3: A) *Rhithrogena hageni* parasitized by *Symbiocladius rhithrogenae* did not have significantly different mass-specific N excretion rates, B) P excretion rates, or C) excretion N:P stoichiometry. Boxes represent the interquartile range (IQR); whiskers represent the 1st and 4th quartiles; the middle horizontal line in each box represents the 2nd quartile, or median; the diamond in each box represents the mean, and circles represent outliers calculated as 1.5*IQR. Asterisks identify significant differences.

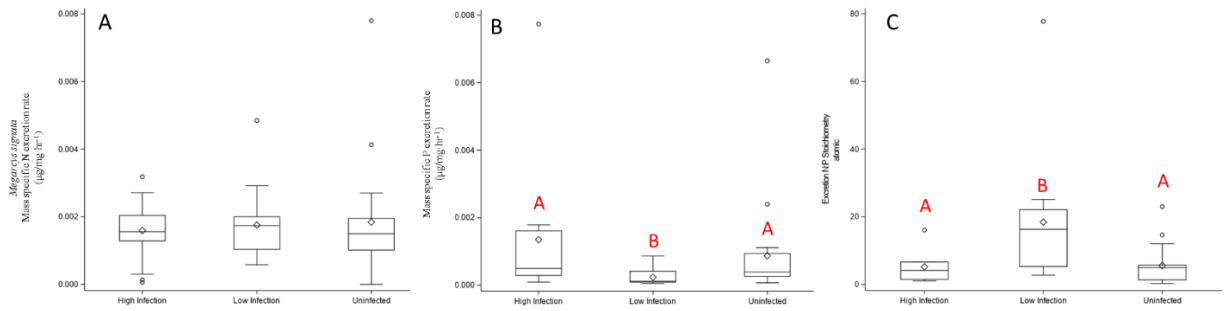

Figure 3.4: A) *Hydrachnidia* mite load was not significantly related to *Megarcys signata* mass-specific N excretion rates B) *Hydrachnidia* infection was, however, related to mass-specific P excretion rates. C) The effects of the parasite on P excretion were evident as a significant change in excretion N:P stoichiometry. Groups with different letters were significantly different. Boxes represent the interquartile range (IQR); whiskers represent the 1st and 4th quartiles; the middle horizontal line in each box represents the 2nd quartile, or median; the diamond in each box represents the mean, and circles represent outliers calculated as 1.5*IQR. Asterisks identify significant differences.

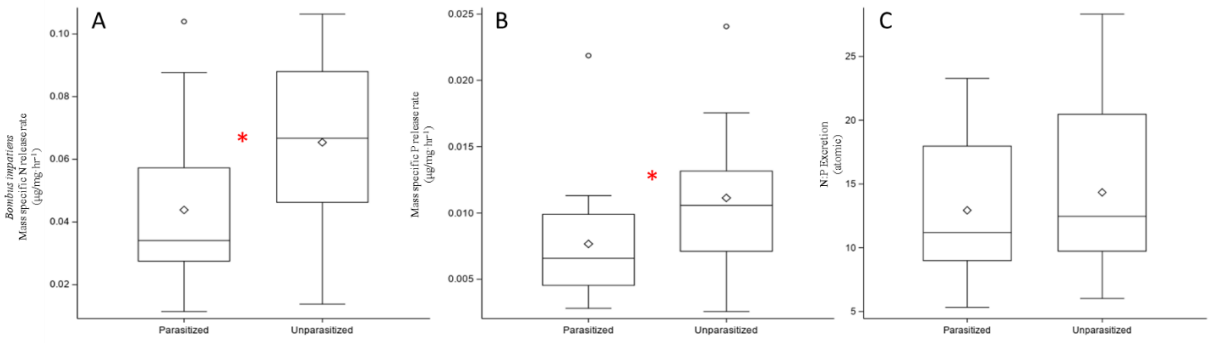

Figure 3.5: A) *Bombus impatiens* parasitized by *Crithidia bombi* did not have significantly different mass-specific N excretion rates compared to unparasitized individuals B) However, unparasitized individuals did have significantly higher mass-specific rates of P excretion. C) Differences in mass-specific P excretion were not evident in excretion N:P stoichiometry. Boxes represent the interquartile range (IQR); whiskers represent the 1st and 4th quartiles; the middle horizontal line in each box represents the 2nd quartile, or median; the diamond in each box represents the mean, and circles represent outliers calculated as 1.5*IQR. Asterisks identify significant differences.

Table 3.1: Summary of results

Host / Parasite Pair	N excretion	P Excretion	N:P Excretion stoichiometry	Tissue C:N:P
Epeorus longimanus / Gasteromermis sp.	Parasitized individuals had higher mass specific N excretion rates than unparasitized individuals	Parasitized individuals had higher mass specific P excretion rates than unparasitized individuals	Excretion stoichiometry was not significantly different between parsitized and unparasitized individuals	Parasitized: 214:39:1 Unparasitized: 178:30:1 Parasite: 384:49:1
Baetis bicaudatus / Gasteromermis sp.	Mass-specific N excretion rate significantly decreased with increasing Gasteromermis infection rate for *B. bicaudatus*	Mass-specific P excretion rate was negatively correlated with increasing infection rate	Excretion N:P stoichiometry increased significantly with infection rate	Parasitized: 291:47:1 Unparasitized: 407:73:1 Parasite: 193:27:1
Rhithrogena hageni / Symbiocladius rhithrogenae	Parasitized *R. hageni* did not have significantly different mass-specific N excretion rates than unparasitized individuals	Mass-specific P excretion rates of parasitized individuals were not significantly different than that of unparasitized individuals	Excretion N:P presented a non-statistically significant trend of higher excretion N:P by unparasitized *R. hageni*	Parasitized: 294:51:1 Unparasitized: 284:47:1 Parasite: 264:37:1
Megarcys signata / Hydrachnidia sp.	No statistically significant effects of Hydrachnidia infection on M. signata N excretion rates were observed	Mass-specific P excretion rates were significantly lower for low burden individuals than high burden and unparasitized individuals	Excretion N:P stoichiometry was significantly higher for low burden individuals compared to high burden and unparasitized individuals	Parasitized: 144:23:1 Unparasitized: 160:27:1 Parasite: No Data
Bombus impatiens / Crithidia bombi	Mass specific N excretion rate was significantly lower for parasitized bees	Mass specific P excretion rate was significantly lower for parasitized bees	Excretion N:P was not significantly different between parasitized and unparasitized bees	Parasitized: No Data Unparasitized: No Data Parasite: No Data

CHAPTER 4: Climate change and trout on the Qualla Boundary

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KEYWORDS: Climate change, trout, tribal, habitat

ABSTRACT

Surface air temperatures are expected to warm by 2-5°C (3.6-9°F) by the year 2100. For coldwater fish in Southern Appalachia that already experience temperatures near their upper limit for growth, climate warming could have severe impacts on their health and distribution. Trout are an important driver of the tourism based economy on the Qualla Boundary, and culturally relevant to the Eastern Band of Cherokee Indians (EBCI). In this study I modeled how standard future climate scenarios might be expected to impact the distribution of native Appalachian brook trout on the Boundary, as well as how climate warming may impact production of fish in the EBCI tribal hatchery. Model predictions suggest that even mild climate warming will result in a significant decrease in brook trout habitat. Bioenergetics models for rainbow trout suggest that even the most extreme climate warming scenario will have relatively minor impacts on the production of fish in the tribal hatchery. These results agree with previous studies at the regional scale of the Southern Appalachians and the overall Appalachians, as well as a study from Northern Europe. This work expands the discussion on brook trout and climate warming in Appalachia by focusing on implications for indigenous peoples who value brook

trout for cultural, economic, and other reasons, but whose present land base may not sustain brook trout habitat in the future because of projected climate change.

INTRODUCTION

The Qualla Boundary is a parcel of 83 sq. miles (214 sq. km) of sovereign Cherokee territory in the mountains of Western North Carolina. It is home to the majority of the members of the Eastern Band of Cherokee Indians (EBCI) (Eastern Band of Cherokee Indians Public Health and Human Services 2018). Brook trout (*Salvelinus fontinalis*) are the only salmonids native to Southern Appalachia, and have always been an important source of protein for the Cherokee people in this region (Altman 2006).

Cherokee, NC, the largest town on the Qualla Boundary, and the seat of the tribal government, is located at a latitude of 35.5 °N, near the southern limit of brook trout distribution (Meisner 1990). There is no seasonal snowpack. Streams in this region are rain and groundwater fed (Kokkonen, et al. 2003) and regularly reach temperatures above the thermal optimum for trout during the summer months (Chadwick and McCormick 2017, USGS 2018).

Because temperature decreases with elevation, species in mountainous regions are expected to shift their ranges upslope in response to climate warming (Chen, et al. 2011). Species that already have ranges extending to the very highest elevations are expected to lose habitat and potentially go extinct (Shoo, et al. 2005). Brook trout in the Southern Appalachians already inhabit the highest elevation streams, and the lower elevation of their range is limited by stream temperature. Thus, climate warming is expected to significantly reduce the amount of habitat suitable for brook trout in Southern Appalachia (Flebbe 1993, Flebbe, et al. 2006, Meisner 1990).

In this study, I modeled how brook trout habitat will be impacted by climate warming under two of the four climate scenarios adopted by the Intergovernmental Panel on Climate Change (IPCC) for its fifth assessment report (AR5) (Meyer, et al. 2014). More specifically, I modeled how increasing groundwater temperatures will lead to upslope shifts in the lower elevational limit of brook trout habitat, and how warming stream temperatures will increase the metabolic (and food) demands of rainbow trout in hatchery raceways.

METHODS

Temperature estimates

Daily mean surface air temperature predictions were generated from the World Climate Research Programme's Coupled Model Intercomparison Project phase 5 (CMIP5) multi-model dataset, using the locally constructed analogs (LOCA) method (Pierce, et al. 2015, Pierce, et al. 2014), and were obtained from the Lawrence Livermore National Laboratory archive (Lawrence Livermore National Laboratory 2018). Mean predictions and confidence intervals were generated from the variation in the temperature predictions of the 16 unique global coupled climate models included in the CMIP5 dataset. The RCPs are named for the radiative forcing value they predict for the year 2100, in W/m^2: RCP4.5 (4.5 W/m^2) and RCP8.5 (8.5 W/m^2). Radiative forcing is the difference between energy absorbed by the Earth and energy emitted back into space by the Earth. Higher radiative forcing values represent more climate warming, and the model predictions for average global surface air temperature increase by the year 2100 for each RCP is as follows: RCP4.5: +2.7 °C (4.9 °F), RCP8.5: +5.1 °C (9.2 °F).

Brook trout habitat models

The lower elevational limit of brook trout distribution is closely correlated with the 15 °C (59 °F) groundwater isocline, which is the lowest elevation where average annual groundwater temperature is 15 °C or cooler (Meisner 1990). We generated estimates for the lower elevational limit of brook trout by modeling how predicted changes in surface air temperature would change the elevation of the 15 °C groundwater isocline. We employed the following model of the 15°C groundwater isocline:

Future LEL = 398 + (188*Δt)

(Flebbe, et al. 2006)

Where "LEL" = "lower elevational limit" in meters, Δt = change in mean annual surface air temperature from today in degrees C, and "lat" = latitude in degrees north. Current LEL was estimated from Meisner (1990) as 500m asl.

To generate representative maps we used ArcGIS ArcMap (ESRI 2015). The elevation data we used were LIDAR data available from the North Carolina Flood Risk Information System (NCFRIS 2018). The vertical accuracy of the LIDAR data was 16.4 ft (5 m). The borders of the Qualla Boundary were obtained from the US Census Bureau (USCB 2010). The perennial streams were mapped from the USGS National Hydrography Dataset (NHD) (USGS 2013). For each time step we clipped the NHD flowlines at the mean elevation and 95 % confidence interval limit elevations output from the models, and calculated the total length of streams in the remaining area.

RESULTS

The brook trout habitat models predicted a loss of 0 km (0 mi). of suitable brook trout habitat in the next 10 years depending on emissions scenario, and a loss of 29 km (11 %, 18 mi.) to 109 km (38.5 %, 68 mi.) of habitat by the year 2068 depending on emissions scenario (figure 1). These results are mapped in figure 4.2a-c(RCP4.5), and figure 4.3a-c (RCP8.5). Each map represents the lower elevational limit of brook trout thermal habitat in a particular year, under a particular scenario, and the 95% confidence interval around the ensemble mean generated from the models in the CMIP5 dataset. The downstream limit of the of the white line represents the lower limit of the 95% confidence interval, and the downstream limit of the black/blue/white line represents the upper limit of the 95% confidence interval, while the downstream limit of the blue/white streams represents the ensemble mean. Grey streams are expected to be too warm for brook trout (outside the 95% confidence interval; below the lower elevational limit for brook trout) and black/blue/white streams are expected to stay cool enough to support brook trout (outside the 95% confidence interval; above the lower elevational limit for brook trout). Therefore the white and blue/white streams represent the elevations where thermal suitability for brook trout cannot be confidently predicted.

Under both scenarios, no stream reaches fall below the 95% confidence interval until year 2068 (grey streams on maps). In 2068, under RCP4.5, 42.8% of 4^{th} order reaches are lost, 40.0% of 3^{rd} order reaches are lost, 23.8% of 2^{nd} order reaches are lost, and 0% of 1^{st} order reaches are lost. Under RCP8.5, 100% of 4^{th} order reaches, 80% of 3^{rd} order reaches, 64.3% of 2^{nd} order reaches, and 47% of 1^{st} order reaches are lost by 2068.

With the loss of such substantial fractions of the higher order stream reaches, habitat connectivity is severely reduced by the year 2068. Under RCP4.5, 11.4% of confluences fall

below the 95% confidence interval, and under RCP8.5, 43% of confluences fall below the 95% confidence interval.

DISCUSSION

The habitat model results suggest that brook trout on the Qualla Boundary are highly vulnerable to the effects of climate warming because of projected increases in groundwater temperature and therefore, stream temperatures. Not only was total habitat reduced under all scenarios, the rugged nature of the terrain in this area resulted in severe habitat fragmentation as well. Perhaps unsurprisingly, these results were similar to those found by Meisner (1990) at the regional level. As the highest elevation perennial stream on the Boundary, Rough Branch is predicted to be the most persistent brook trout habitat.

Total habitat loss by 2068 is expected to be 11-38.4%, however, the majority of lost habitat is 3rd and 4th order streams, which were lost at much higher percentages. In Southern Appalachia, brook trout have generally been extirpated from these streams by invasive rainbow and brown trout (Davis *et al.* 2015), but if barriers to movement are not present, can be important thoroughfares between the lower order streams where most brook trout reside (Anglin and Grossman 2013, Mollenhauer *et al.* 2013). Indeed, genetic connectivity between brook trout populations has been shown to rely on mainstem river habitat considered unsuitable for year-round brook trout occupancy (Nathan *et al.* 2019), highlighting both the importance of these habitats to brook trout genetic diversity, and the possibility that warming in these reaches may not impact their role in brook trout meta-population health and persistence. Thus, the impacts of climate change on brook trout populations in Southern Appalachia, may or may not be more

severe than that predicted by the loss of gross habitat alone, but depends on the continued availability of suitable habitat in low order headwater reaches.

The habitat models used in this study do not account for climate change impacts other than increasing groundwater temperatures, including loss of tree canopy or changes in precipitation, both of which may influence stream temperature. Loss of shading from tree canopy, for example like that caused by Hemlock Wooly Adelgid in Southern Appalachia (Snyder, et al. 2002, Vose, et al. 2013), would lead to warmer than predicted stream water and could interfere with the relationship between habitat suitability and the 15°C groundwater isocline. Total annual precipitation in the North Carolina Appalachians is expected to increase moderately with climate change, but is expected to arrive as fewer, more severe events (Swain and Hayhoe 2015), and recent studies of historical precipitation patterns at the Coweeta Hydrologic Laboratory in Macon County, NC support these prediction (Burt T. P., et al. 2018). Greater precipitation could lead to less habitat loss than predicted (Merriam, et al. 2017), but drought periods between rain events could result in less suitable habitat during the summer months when stream levels are already low. Receiving rain in fewer, more severe events would also result in more of the precipitation running off in flood events and less groundwater recharge, reducing base flow and potentially reducing the strength of the correlation between habitat suitability and the 15°C groundwater isocline (Loheide and Gorelick 2006).

Implications for indigenous peoples

RCP8.5 is presented in this study as the "worst case scenario". Some studies have suggested that RCP8.5 overestimates the availability of fossil fuels, meaning that it is an overly pessimistic model in the long term (Wang, et al. 2017). However, other recent studies on

greenhouse gas emissions indicate that we are currently overshooting RCP8.5, meaning that it may be overly optimistic for shorter timescales (Sanford, et al. 2014). Comparatively, RCP2.6 represents the goal of the 2015 Paris Climate Agreement, and represents the "best case scenario". Predictions for RCP2.6 were not presented in this study because RCP2.6 was not included in the CMIP5 project. RCP4.5 was presented in this study as the low-moderate climate change scenario. By discussing the effects of climate change in the context of these potential scenarios, the EBCI can create adaptation plans that are robust to the range of possible climate futures the tribe faces.

However, because the EBCI's current land base is a small fraction of their former sovereign territory, the tribe's ability to adapt to climate change is also reduced. For example, more northern areas of Cherokee territory (parts of modern day NC, TN, and VA) that could be refuges for trout in a warmer future are not currently subject to tribal decision making, meaning tribal members would require state licenses to fish there, possibly losing access to a culturally important subsistence foodstuff, and the tribe would not be able to reap the same economic benefit from fish in those areas that they enjoy currently. Further, climate change is primarily a result of industrialization in large wealthy nations. It is a problem that was not caused by, nor can hardly be solved by, the 14,000 members of the tribe. Though perhaps not as severely existential as the climate warming impacts faced by coastal tribes like the Isle de Jean Charles Band of Biloxi-Chitimacha-Choctaw Indians and the Yup'ik, or Pacific Island peoples like the Chamorro (Maldonado, et al. 2013), the cultural and economic impacts of climate change on the EBCI presented here are another example of climate and social injustice faced by indigenous peoples (Tsosie 2007). These climate injustices follow a long history of removal and intergenerational

trauma imposed upon indigenous peoples, and represent the most recent iteration of what Kyle Whyte refers to as "colonial déjà vu" (Whyte 2016).

Traditional knowledge and "indigenuity" are powerful tools for adaptation and have served indigenous people well, however western science can be a force multiplier if motivated and led by indigenous communities and their own specific priorities (Wildcat 2013). Collaborations with indigenous communities and traditional knowledge can be useful for western science as well (e.g. Bonta, et al. 2017, Zakrzewski 2002). Unfortunately, the majority of scientific research involving indigenous communities is exploitative rather than indigenous or collaborative (David-Chavez and Gavin 2018). We thus implore researchers in the academy to seek out opportunities to work with indigenous communities and to do so on their terms.

Conclusion

To conclude, the results of this study suggest that climate warming will have a severe impact on the native fishes and stream ecosystems of the Qualla Boundary. It does however make several assumptions and is a simplification of a large and complex system. The assumptions included in the models likely make them somewhat optimistic.

Overall, the study sought to present a range of climate futures that can aid in decision making and generate further, deeper inquiries into the predicted impacts of climate change on the members of the Eastern Band of Cherokee Indians and the unique culture, ecology and economy of the Qualla Boundary. This study should also stand as another case of climate injustice experienced by indigenous people, and an example of the application of academic science to

indigenous issues in collaboration with indigenous communities and guided by indigenous priorities.

ACKNOWLEDGEMENTS

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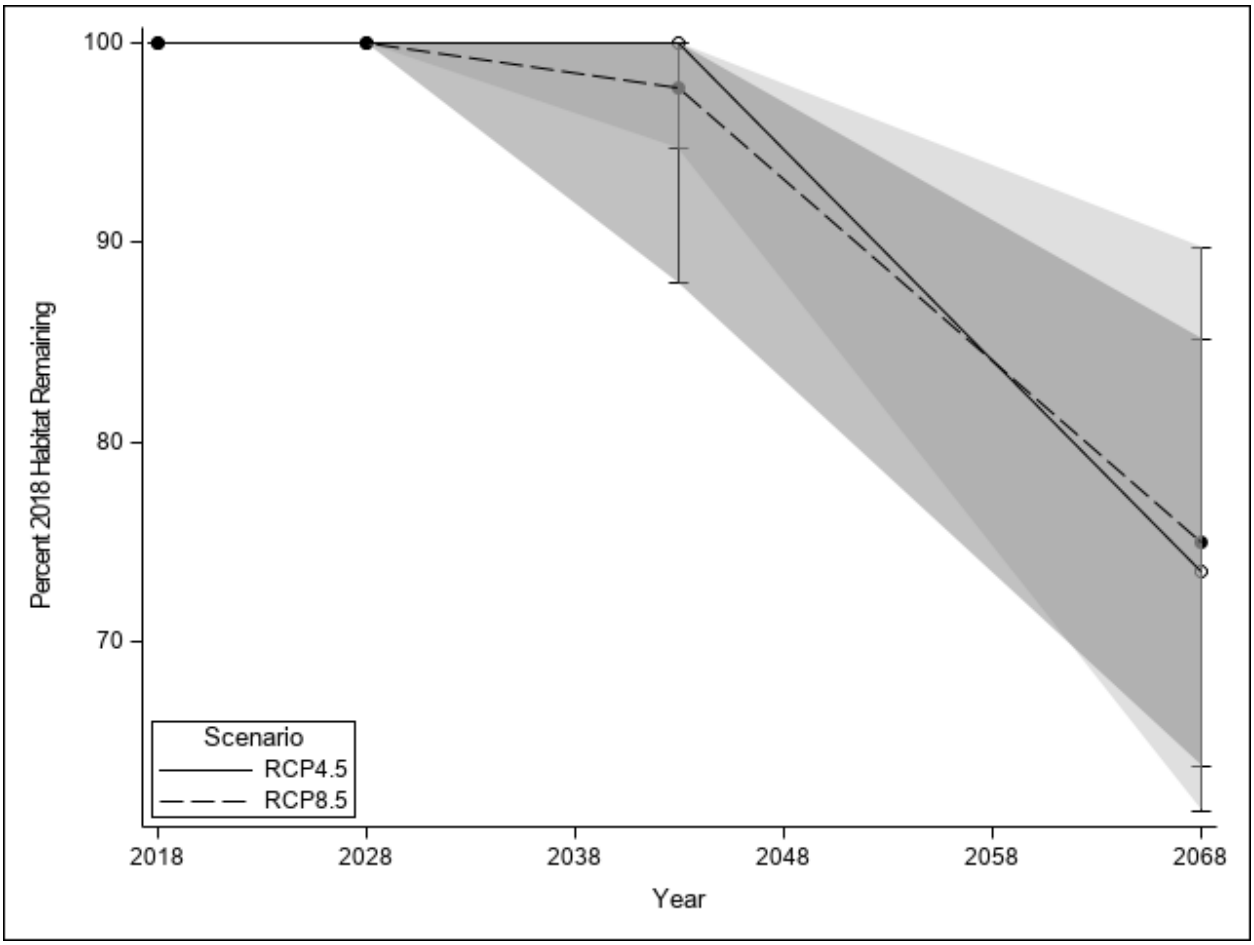

Figure 4.1: Summary of model outputs for percent of habitat lost over time for each of the RCP climate scenarios. Bands represent 95% confidence intervals.

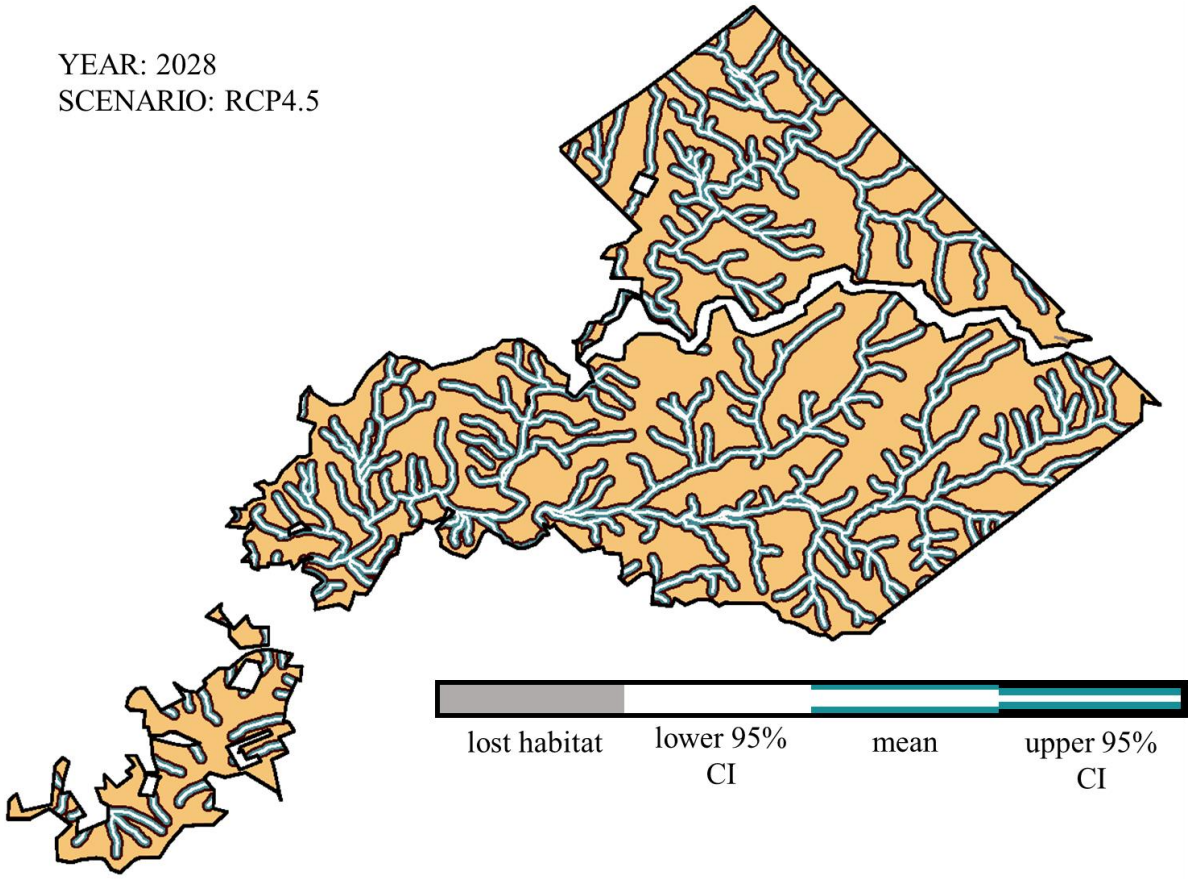

Figure 4.2a: Predicted extent of Appalachian brook trout on EBCI land in the year 2028, under scenario RCP4.5

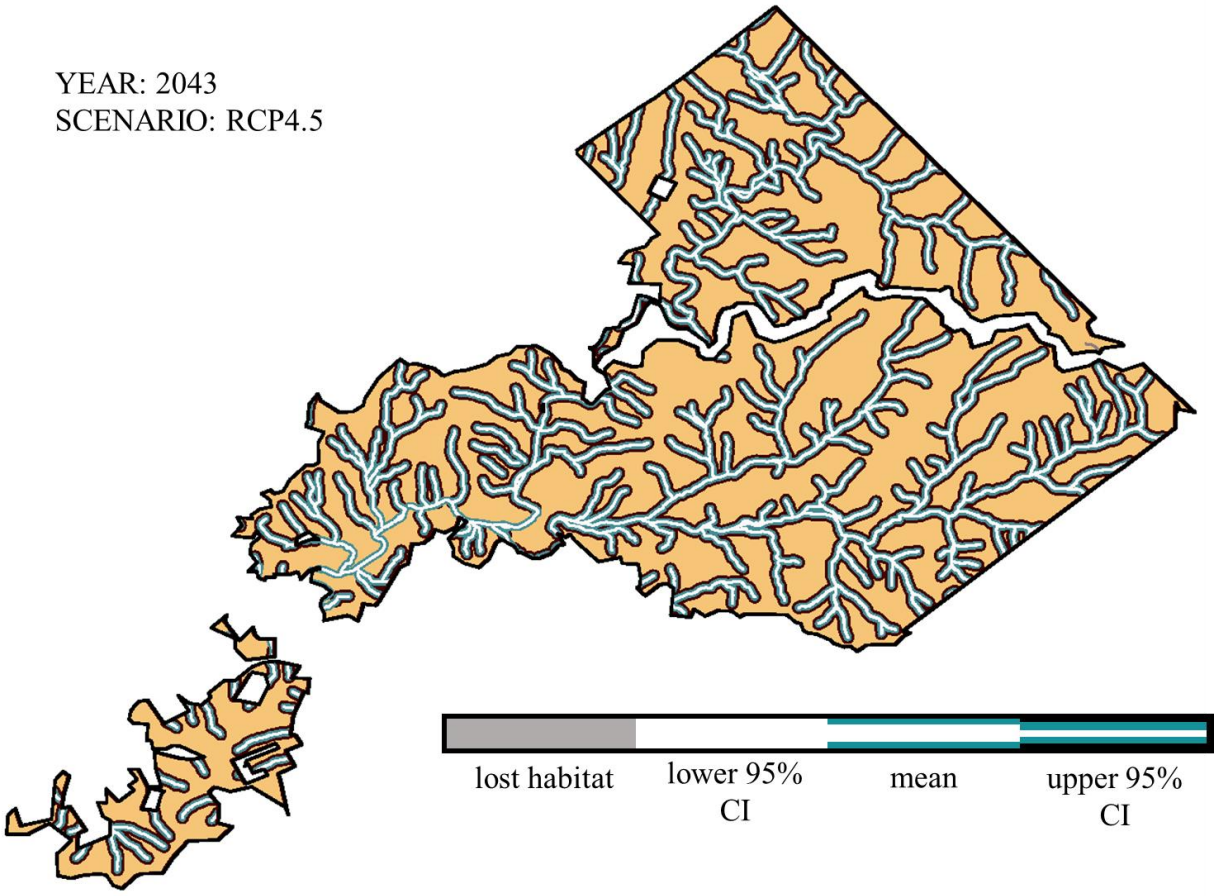

Figure 4.2b: Predicted extent of Appalachian brook trout on EBCI land in the year 2043, under scenario RCP4.5

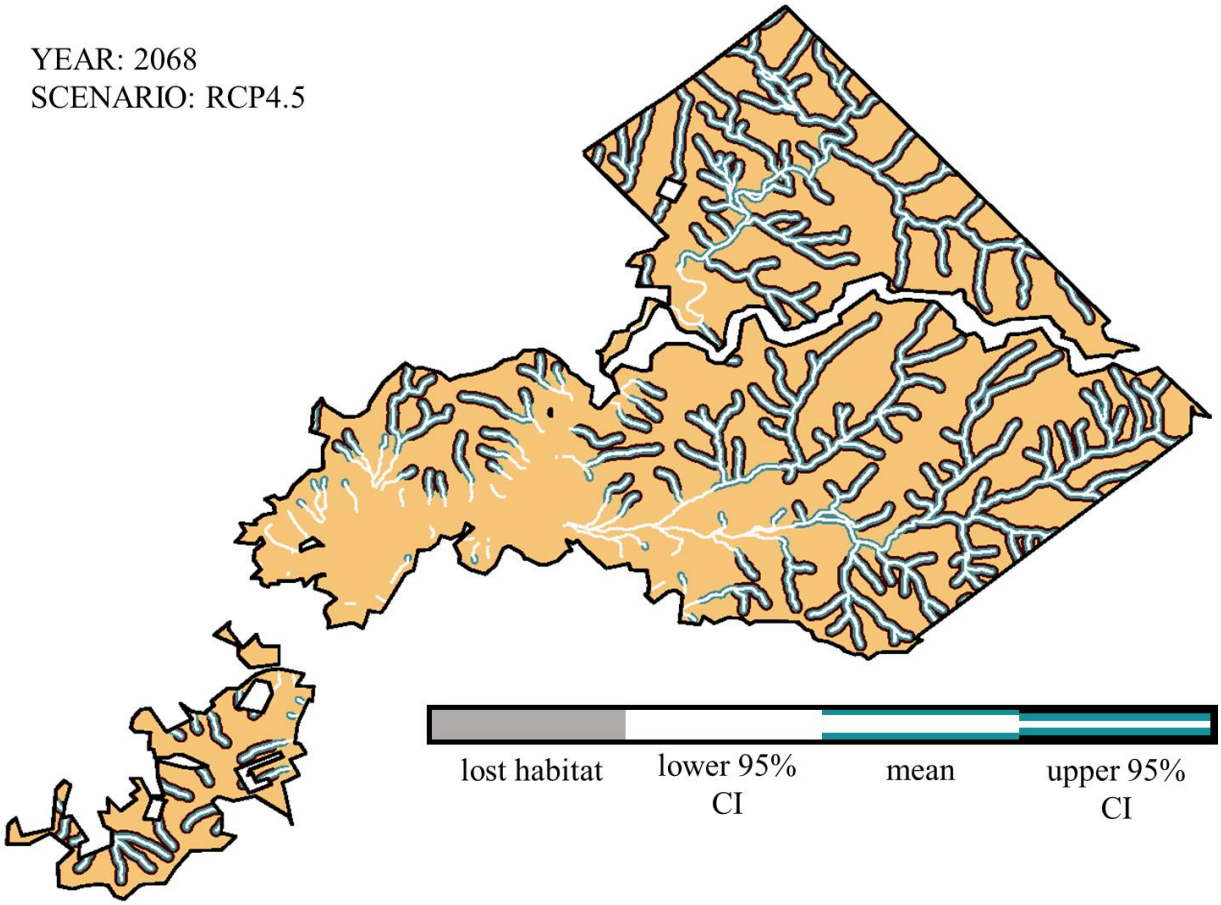

Figure 4.2c: Predicted extent of Appalachian brook trout on EBCI land in the year 2068, under scenario RCP4.5

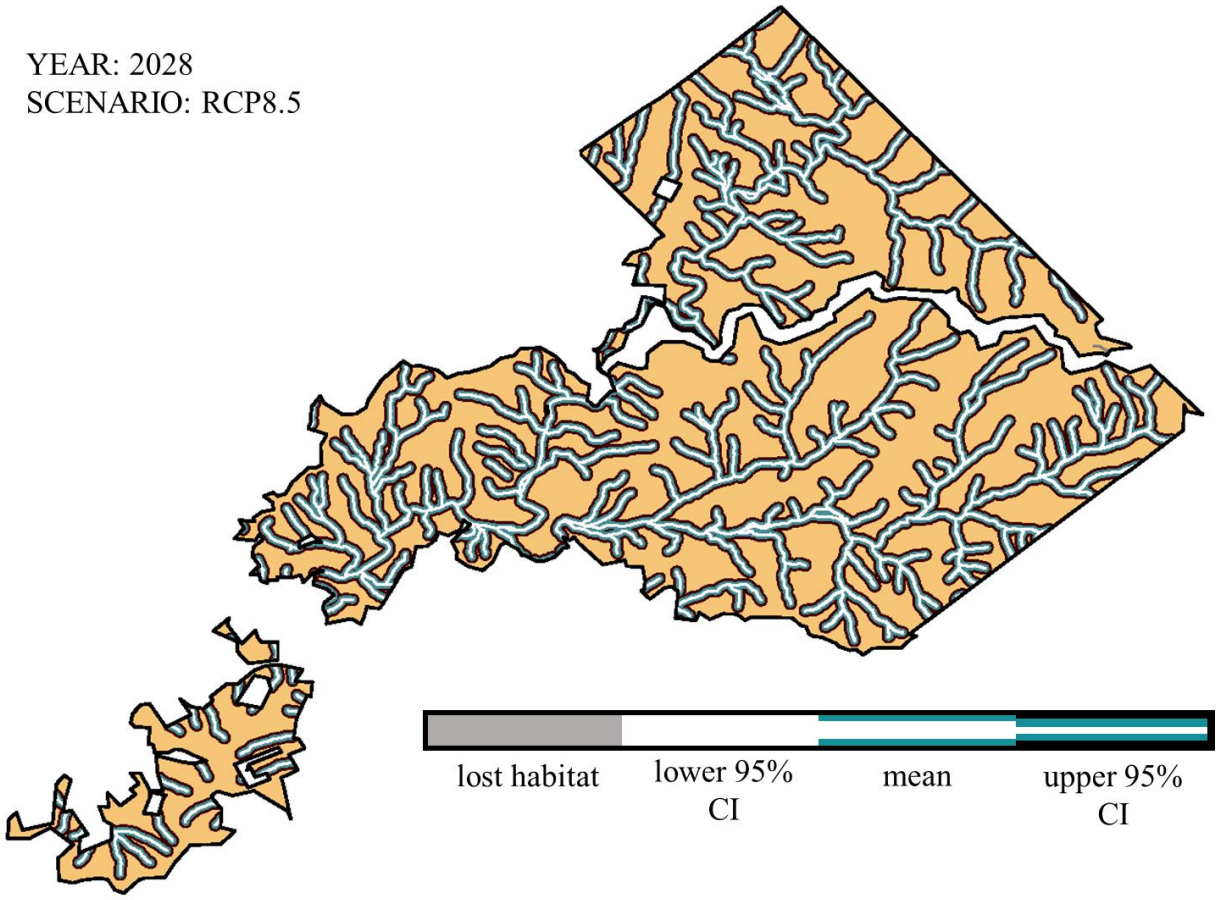

Figure 4.3a: Predicted extent of Appalachian brook trout on EBCI land in the year 2028, under scenario RCP8.5

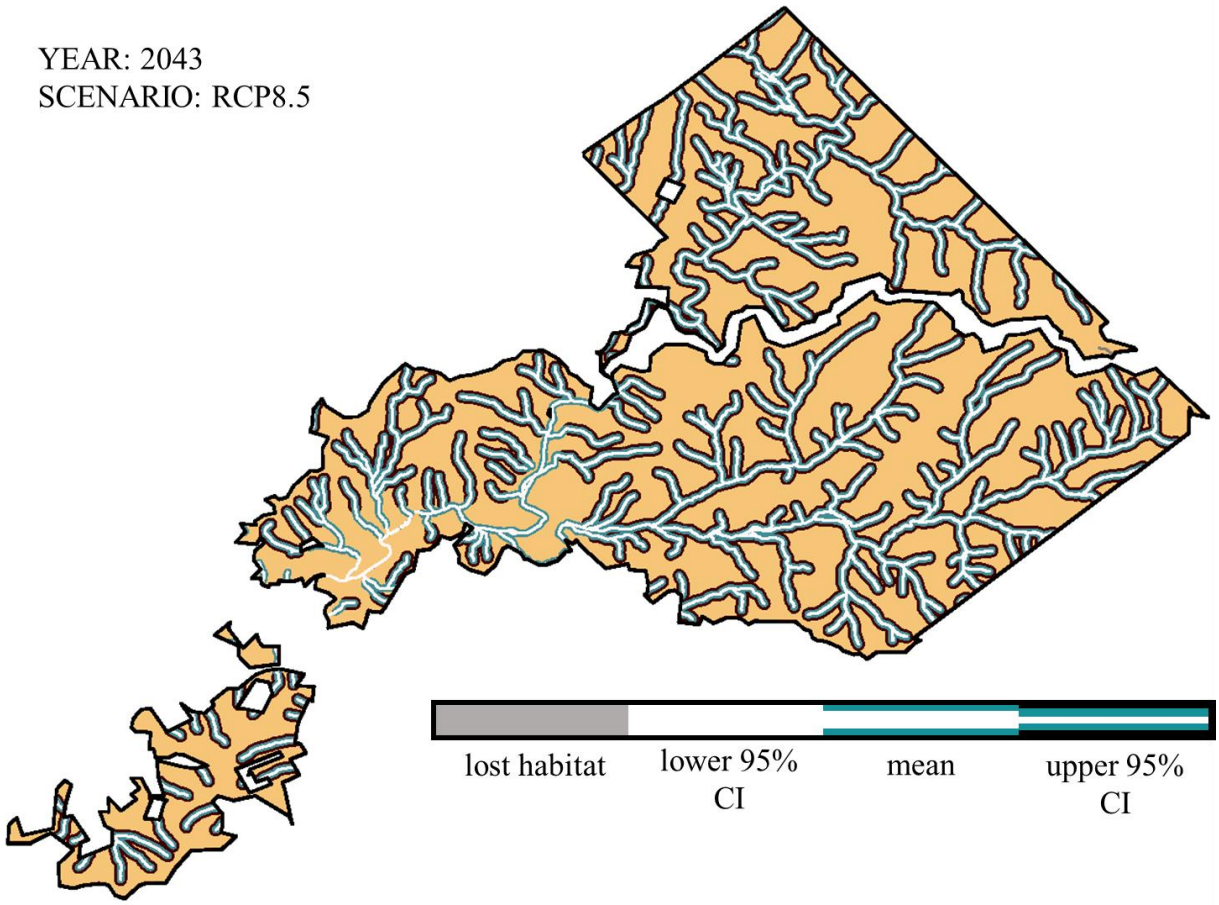

Figure 4.3b: Predicted extent of Appalachian brook trout on EBCI land in the year 2043, under scenario RCP8.5

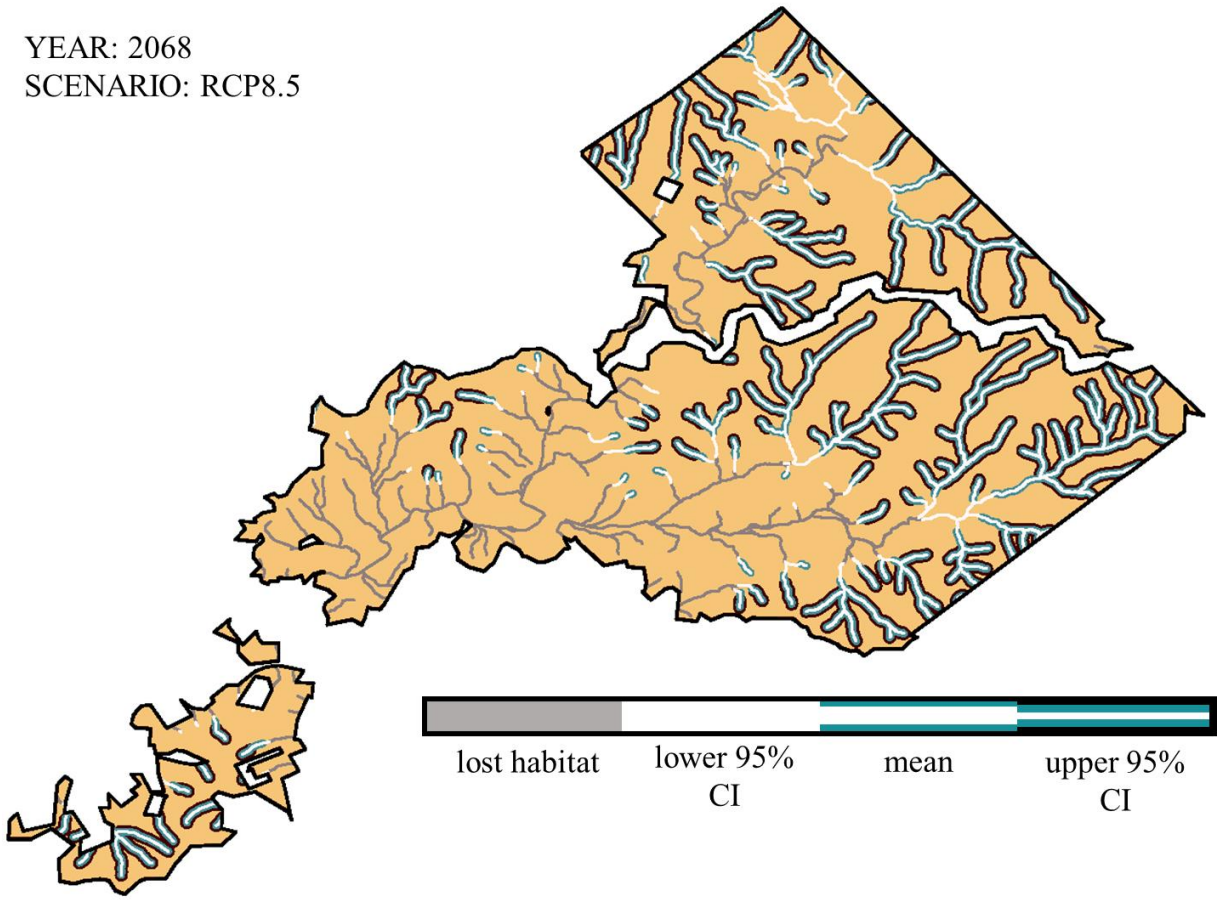

Figure 4.3c: Predicted extent of Appalachian brook trout on EBCI land in the year 2068, under scenario RCP8.5

APPENDIX

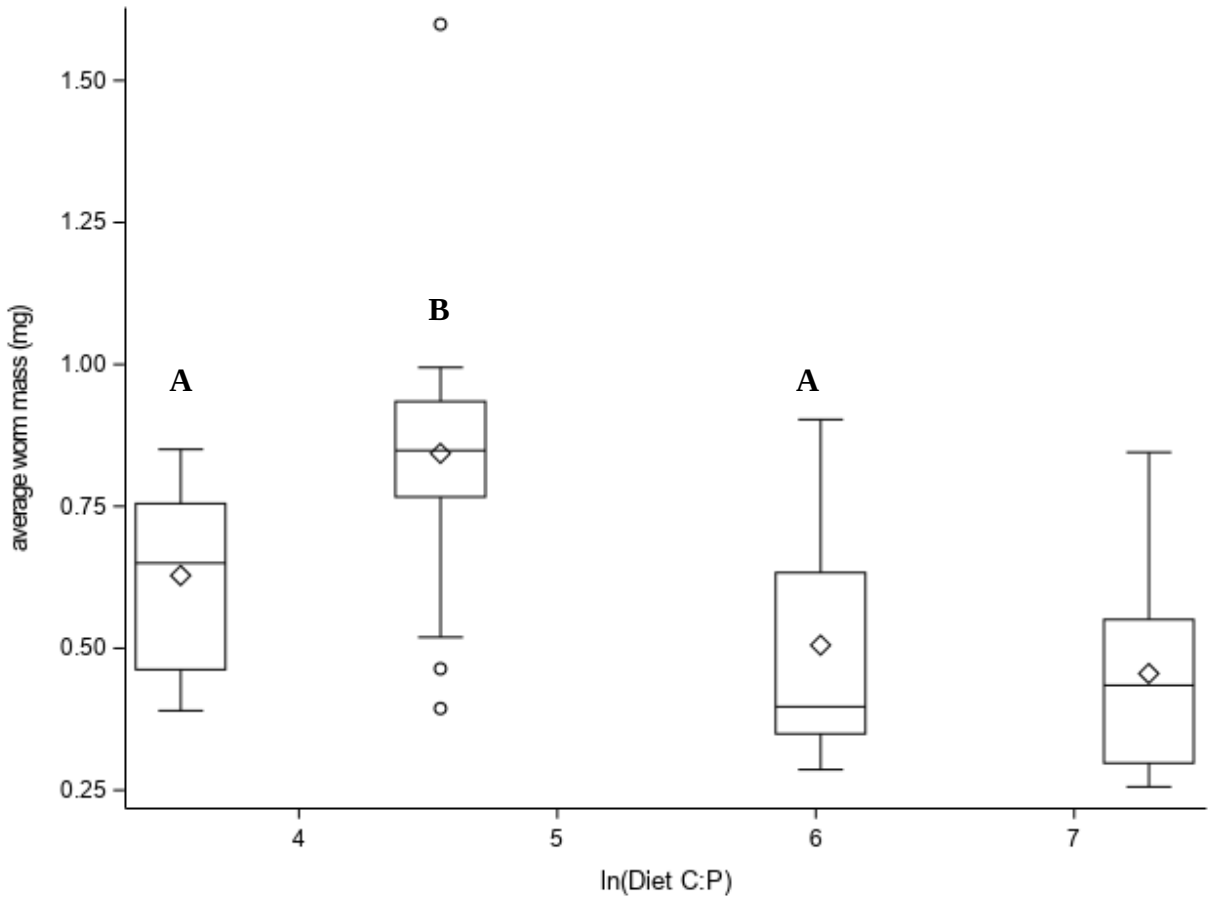

Figure S2.1: Worms fed the lnC:P = 4.6 were significantly heavier at the end of the experiment than worms fed the other diets. ANOVA, n = 63, $F_{3,59} = 10.95$, $p < 0.01$. Removal of the high outlier in the lnCP = 4.6 diet did not qualitatively change the conclusion of the statistics. Box represents inter-quartile range, line in box represents median, bars represent maximum and minimum values (without outliers), open circles represent outliers (calculated as >1.5*IQR), and open diamond represents the mean.

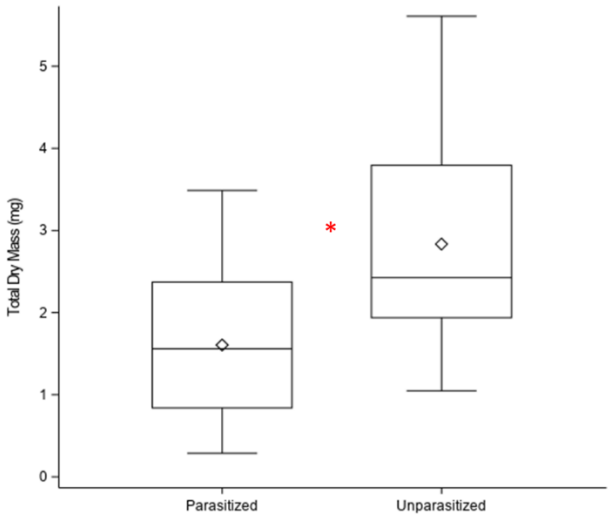

Figure S3.1: Unparasitized *Epeorus longimanus* had significantly greater dry mass than parasitized *E. longimanus* including *Gasteromermis* dry mass. Box represents inter-quartile range, line in box represents median, bars represent maximum and minimum values (without outliers), open circles represent outliers (calculated as >1.5*IQR), and open diamond represents the mean.

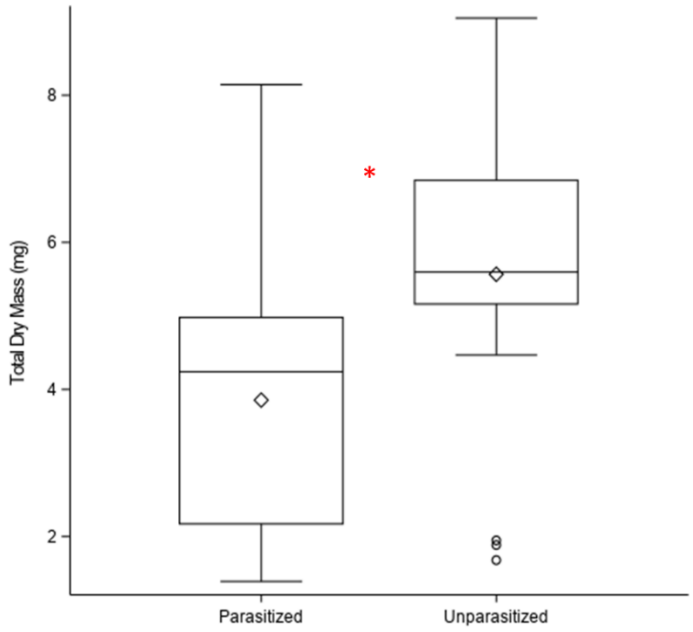

Figure S3.2: Parasitized *Rhithrogena hageni* had significantly less dry mass (including *Symbiocladius rhithrogenae* dry mass) that unparasitized individuals. Box represents inter-quartile range, line in box represents median, bars represent maximum and minimum values (without outliers), open circles represent outliers (calculated as >1.5*IQR), and open diamond represents the mean.

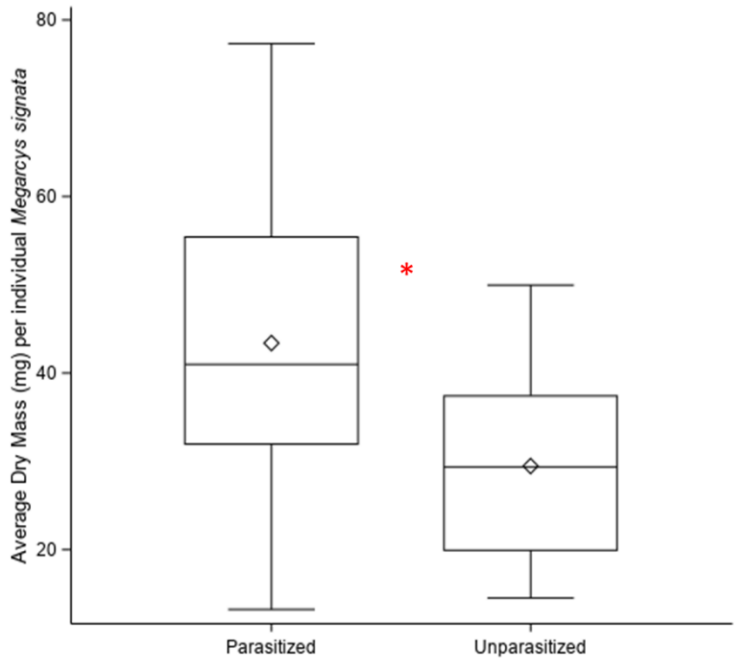

Figure S3.3: Parasitized *Megarcys signata* were significantly larger than unparasitized individuals Box represents inter-quartile range, line in box represents median, bars represent maximum and minimum values (without outliers), open circles represent outliers (calculated as >1.5*IQR), and open diamond represents the mean.