Predation on parasites and its consequences for transmission

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PREDATION ON PARASITES AND ITS CONSEQUENCES FOR TRANSMISSION

by

Sarah Anne Orlofske

B.S. University of Wisconsin – Stevens Point, 2006

A thesis submitted to the
Faculty of the Graduate School of the
University of Colorado in partial fulfillment
Of the requirement for the degree of
Doctor of Philosophy

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This thesis entitled:
Predation on parasites and its consequences for transmission
Written by Sarah Anne Orlofske
has been approved for the Department of Ecology and Evolutionary Biology

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The final copy of this thesis has been examined by the signatories and we
Find that both the content and the form meet acceptable presentation standards
Of scholarly work in the above mentioned discipline.
Predation and parasitism are each important ecological processes within communities and ecosystems. However, the interactions between predation and parasitism may have significant consequences for transmission dynamics and disease in host populations. Predators play multiple roles including changing host densities, behavior, and morphology that could lead to different disease outcomes. Furthermore, direct predation on parasites can potentially lead to reduction in disease risk to host populations. Here, I used a combination of mathematical modeling, small-scale laboratory studies, and a semi-realistic mesocosm experiment to characterize parasite transmission dynamics, provide a predictive framework for the role of direct predation on parasites, and evaluate the direct and indirect (trait-mediated interactions) effects of multiple predators on transmission and infection. My study system included the trematode parasite, *Ribeiroia ondatrae*, larval amphibian hosts, and a suite of invertebrate and vertebrate species that co-occur in nature. Using laboratory experiments and maximum likelihood approach, I characterized non-linear functions as the most accurate representation of transmission. These models capture the saturation of infection at high exposure levels and subsequent experiments
with anesthetized hosts suggest that parasite behavior may be an underlying mechanism for non-linear relationships. Next, I identified damselfly larvae and juvenile mosquitofish (*Gambusia affinis*) as predators of *R. ondatrae* and California Newts (*Taricha torosa*) as alternative hosts. In transmission trials, both damselflies and newts reduced transmission by ~50% through the independent mechanisms of consumption and infection. Additional experiments including a wider range of parasite species showed that predation on parasites is based on predator foraging mode (active vs. ambush) and body size, parasite size and light availability. In more realistic aquatic communities with multiple trophic levels, I found that the trait-mediated indirect effects of predators of parasites and predators of hosts, including reduced activity and morphological changes were associated with higher infection compared to communities with predators absent. My research demonstrates both the direct and trait-mediated roles of predation on disease dynamics and identifies direct predation on parasites as an important factor in transmission dynamics. Future research should evaluate patterns of predator diversity and abundance with the prevalence and pathology due to *R. ondatrae* in nature.
DEDICATION

This thesis is dedicated to my parents who inspired me to love nature and supported me in my scientific adventures, for my sister who has been a source of encouragement in the face of numerous adversities, and my husband who has shown his passion and love for me and biology through so many hours in the laboratory, the field, and the completion of manuscripts.
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Chapter 1
Integrating Predator and Disease Ecology

Diseases of both humans and wildlife are emerging worldwide concurrently with accelerating biodiversity loss (Daszak et al. 2000, Sala et al. 2000). In some cases, biodiversity may protect against infectious disease through the “dilution effect”, which states that community diversity -- including host and non-host species -- reduces pathogen transmission by reducing densities or encounter rates between infected hosts or vectors (LoGiudice et al. 2003, Keesing et al. 2006, Swaddle and Calos 2008, Ostfeld 2009). Communities supporting different species compositions or relative abundances of alternative hosts or predators may have lower levels of infection (Dobson et al. 2006, Johnson and Thieltges 2010). Foundational to the study of the diversity-disease relationship are accurate descriptions of pathogen transmission among hosts (McCallum et al. 2001, Fenton et al. 2002). We can use mathematical models describing transmission dynamics to predict how members of the community, including host and non-host species, may enhance or interfere with transmission (Dobson 2004, Rudolf and Antonovics 2005).

Predators in particular can significantly alter disease dynamics amongst many other ecosystem functions (Packer et al. 2003, Pongsiri et al. 2009). Previous research on the roles of predation in community and ecosystem functions has focused on changes in prey densities through lethal interactions (Peckarsky et al. 2008). Similarly, the relationship between predation and pathogen transmission has been investigated in terms of predator-driven reduction in host densities, leading to a decrease in transmission (Packer et al. 2003, Ostfeld and Holt 2004, Borer et al. 2009). Beyond these previously studied effects, predators can also alter transmission by directly reducing the abundance of free-living infective stages through consumption (Johnson et
al. 2010, Johnson and Thieltges 2010). Finally, predators may also exert indirect effects through trait-mediated changes on host behavior and morphology, both of which may have important consequences for infection and pathology (Thiemann and Wassersug 2000, Decaestecker et al. 2002, Szuroczki and Richardson 2012).

I begin this chapter with an overview of the forms of pathogen transmission and the associated consequences for host populations. Then, I discuss the role of community diversity in mediating transmission. Next, I extend predator-prey theory to predation on parasites and examine the role of trait-mediated interactions on disease. Finally, I provide an overview of my dissertation, which aims to incorporate predator and parasite-host interactions to improve our understanding of the mechanisms through which species diversity affects disease dynamics.

Transmission as a foundation for understanding disease dynamics

Transmission is a major requirement for predicting the impact of a pathogen on a host population (Hone et al. 1992, Greer et al. 2008). As a result, it has become the key factor in theoretical models of disease dynamics (McCallum et al. 2001). By understanding the mathematical form of transmission it is possible to predict whether a pathogen will regulate a host population or respond to a particular control measure (McCallum et al. 2001). The specific form of transmission also determines the potential of a parasite to cause host extinction or of other species in the community to interfere with transmission (Dobson 2004, de Castro and Bolker 2005, Keesing et al. 2006). However, there is still a limited amount of experimental and observational data supporting particular modeling approaches (McCallum et al. 2001).

In most host-pathogen models transmission is assumed to be density-dependent (McCallum et al. 2001). Conceptualization of this form of transmission originally comes from
the work of Kermack and McKendrick (1927), who based their theoretical models of pathogen transmission on the mass action equations of chemical reactions where the rate of the reaction between two chemical species is proportional to the product of their respective concentrations (D’Amico et al. 1996). For pathogen transmission, density-dependence indicates that the probability of a susceptible individual (S) becoming infected is a function of the density of infective individuals (I), with the transmission coefficient represented by $\beta$ (Antonovics et al. 1995). This transmission coefficient, $\beta$, is a per capita rate that is also called the ‘force of infection’ because in addition to representing the rate of contacts between infected and susceptible individuals it also includes the probability of infection due to that contact (Begon et al. 2002).

Density-dependent transmission has increasingly been contrasted with frequency-dependent transmission (McCallum et al. 2001, Begon et al. 2002). Frequency-dependent transmission is characterized as the probability of a susceptible becoming infected as a function of the proportion (rather than abundance) of infectives. Thus, the key difference between density and frequency dependent transmission is that density dependence assumes that contact rate increases with population density leading to increasing transmission, while frequency dependence assumes a constant rate of contact regardless of host population density. Frequency-dependent transmission is often used to describe vector borne or sexually transmitted diseases (Antonovics et al. 1995). These two functional forms make different predictions for the role of community diversity in pathogen transmission. For example, higher host diversity would lead to lower infection for a pathogen with frequency-dependent transmission, whereas a more diverse community of hosts would lead to an increase in infection for a pathogen with density dependent transmission (Dobson 2004).
Instead of a simple dichotomy between density- and frequency-dependent forms, these functions represent the extreme ends of a continuum (Antonovics et al. 1995, McCallum et al. 2001). Both density- and frequency-dependent transmission forms assume homogenous mixing. Because this underlying assumption is often invalid, epidemiological models with non-linear transmission functions were developed for a variety of systems (Liu et al. 1986, 1987, Hochberg 1991). Other potential mechanisms for non-linear transmission beyond non-homogenous mixing include saturation of infection as host or parasite densities increase, or other sources of host heterogeneity including susceptibility of hosts or contact rates (Hochberg 1991, Briggs and Godfray 1995). These phenomenological models also describe the transmission of parasitoids based on their searching behavior, and perform as well as more mechanistic models (Reeve et al. 1994, Briggs and Godfray 1995). In the few cases where a variety of competing functions have been tested by experimental data, non-linear forms of transmission more accurately describe the dynamics (Thrall and Jarosz 1994, D’Amico et al. 1996, Barlow 2000, Fenton et al. 2002, Greer et al. 2008). These non-linear functions allow for a much wider range of dynamical behavior, including infection outbreaks and collapse, or periodic coexistence of parasite and host (Liu et al. 1986, 1987). Given the divergent predictions of the various functional forms, it is imperative to empirically test transmission functions to accurately predict the effects of pathogens on host populations and the consequences of environmental change, such as changes in biodiversity, that may enhance or interfere with transmission (McCallum et al. 2001, Fenton et al. 2002, Dobson 2004).

*The role of community diversity and parasite transmission*

Frequently, studies in disease ecology have only addressed the interactions between a single host
and a single pathogen (Ostfeld and Keesing 2012). However, parasites and hosts interact within a wider community that is capable of influencing transmission dynamics and leading to altered patterns of infection and pathology. Recently, consideration of how variation in competence—the ability to acquire and transmit infections—among host species within communities affects disease risk has led to the development of the “dilution effect” hypothesis, defined as a reduction in disease risk in more biologically diverse communities (Ostfeld and Keesing 2000a,b, Keesing et al. 2006, Keesing et al. 2010, Ostfeld and Keesing 2012). One of the proposed explanations for diversity-disease relationships is that species-rich communities potentially include a greater number or abundance of species more likely to interfere with transmission (low competence hosts), whereas communities with lower species richness contain species that are more likely to support transmission (high competence hosts) (Ostfeld and Keesing 2000a, Dobson et al. 2006, Keesing et al. 2006). For example, simulation models of Lyme disease show that in communities where species were removed in a random sequence (i.e. random disassembly), increased the density of infected nymphal ticks, a measure of disease risk. In contrast, for communities where disassembly was based on empirical data, communities with higher biodiversity showed reduced disease risk (Ostfeld and LoGuidice 2003). Indeed, a recent empirical study showed that patterns of pathogen transmission correspond to the predictable loss of species that are less competent hosts for infection (Johnson et al. 2013).

Most other empirical studies demonstrating the importance of species composition and relative abundance rely on comparing species richness to proximate measures of disease risk, such as the prevalence of infected vectors (Dobson et al. 2006, Ostfeld 2009). For example, higher host diversity in free-living communities is associated with lower Lyme disease risk and reduced West Nile virus infection (LoGiudice et al. 2003, Swaddle and Calos 2008). However,
emerging research shows that disease risk may not depend strictly on biodiversity (i.e. host richness) per se, but on how species composition changes predictably with diversity (LoGiudice et al. 2008, Johnson et al. 2013).

The correlational nature of many studies of disease makes it unclear which particular species or ecological mechanisms are responsible (Allan et al. 2003, Ezenwa et al. 2006). The occurrence of alternative, low-competency hosts, which reduce the number of successful transmission events, is among the most commonly studied mechanism (Keesing et al. 2006). Other potential mechanisms include reducing the probability of transmission given an encounter or increasing the recovery rate (Keesing et al. 2006). In some cases, multiple mechanisms could operate simultaneously underscoring the importance of evaluating the functional roles of multiple species and assessing how they affect transmission. Importantly, mechanisms beyond reduced encounter by alternative hosts require additional study.

**Predator-prey interactions and predation on parasites**

Besides hosts, other members of the community can influence transmission and infection. In particular, predators of hosts can alter parasite dynamics through a reduction in the density of susceptible individuals or an increase in the death rate of infected individuals (Packer et al. 2003, Ostfeld and Holt 2004, Keesing et al. 2006). For example, predators may indirectly control pathogen exposure by reducing populations of rodent reservoirs or insect vectors (Ostfeld and Holt 2004, Moore et al. 2009, Muñoz-Pedreros et al. 2010). Predation can also lead to infection of the predator if parasites are transmitted trophically (Lafferty 1999, Hall et al. 2007).

A direct mechanism by which predation can alter disease dynamics is through predation on parasites (Johnson et al. 2010, Johnson and Thieltges 2010). Predation on parasites can take a
number of forms including consumption of parasites along with prey or grooming of
ectoparasites (Johnson et al. 2010). Predation may be a particularly important factor influencing
the transmission of macroparasites (e.g. platyhelminths), for which there are free-living infective
stages and pathology is intensity-dependent. If free-living infective stages of parasites are
removed from the environment by predators before they infect a host, then prevalence
(percentage of hosts infected with a particular parasite) and intensity (number of parasites in a
single host) of infections may be altered (Thieltges et al. 2008a,b, Prinz et al. 2009). Recent
evidence from food web studies that incorporate parasites, suggest that predation on parasites
occurs frequently in nature, particularly for taxa with free-living stages such as cestodes,
trematodes, and nematodes (Lafferty et al. 2008, Johnson et al. 2010).

While predation can influence transmission through effects on population dynamics of
both hosts and parasites, predators can also alter host traits such as behavior, susceptibility, or
morphology, also leading to indirect modification of transmission or pathology (Johnson et al.
2006, Keesing et al. 2006, Belden and Wojdak 2011, Duffy et al. 2011). The presence of
predators may reduce host activity including anti-parasite behavior, thereby increasing host
susceptibility to infection (Thiemann and Wassersug 2000, Szuroczki and Richardson 2012).
Alternatively, by investing resources in anti-predator defenses, hosts may have fewer resources
available to mount an effective immune response resulting in higher parasite infection (Ribby
and Jokela 2000, Navarro et al. 2004, Stoks et al. 2006). Given the wide variety of potential roles
of predators on host-parasite interactions, it is important to examine the relative importance of
each in order to better understand the net effect of predators on transmission dynamics in nature.
**Organization of the dissertation**

The question of the situations in which predators are likely to have a significant effect on parasite transmission and pathology is the focus of my dissertation research. To address this question, I use a model system including *Ribeiroia ondatrae*, a multi-host trematode parasitizing amphibians during its larval stage, which has been implicated as a widespread cause of severe amphibian malformations, including missing, extra, and deformed limbs (Johnson et al. 1999, 2002). The Pacific Chorus Frog, *Pseudacris regilla*, serves as my focal amphibian host because it is among the species with the highest reported frequencies of malformations, approaching 90% among newly metamorphosed (juvenile) frogs at some wetlands (Johnson et al. 1999, 2001, 2002). *Ribeiroia ondatrae* has a complex life cycle, sequentially infecting snails of the genus *Helisoma* (first intermediate host), larval amphibians (second intermediate host), and finally amphibian-eating birds (definitive hosts). Transmission of *R. ondatrae* from snails to amphibians occurs through direct infection by free-living aquatic parasite stages known as cercariae, which then form encysted metacercariae in the amphibian. The life cycle of this parasite parallels those of other trematodes, making it useful for addressing general questions in disease ecology. I base my laboratory studies on communities in California wetlands where several other amphibian trematodes co-occur with *R. ondatrae*, including *Echinostoma trivolvis*, *Cephalogonimus americanus* and a distinct morphotype “Magnacauda”. Therefore, I address certain aspects of my research with these other parasite species. In addition to parasites and hosts, the ecological communities in which these interactions take place consist of a wide variety of other species, including potential alternative hosts for *Ribeiroia* such as California newts (*Taricha torosa*), introduced Western Mosquitofish (*Gambusia affinis*), as well as non-host aquatic insect larvae (damselflies and dragonflies), backswimmers, clam shrimp, and clams. Through the course of
my research, I consider the roles that these species play in transmission dynamics of *R. ondatrae* cercariae to their amphibian hosts.

My thesis begins with laboratory studies investigating the transmission mode of macroparasites (Chapter 2). I investigate which mathematical function best represents the transmission dynamics of free-living infective stages of trematode parasites. Using a model fitting approach based on maximum likelihood, I examine a suite of seven mathematical functions representing transmission including classical density and frequency dependence and non-linear functions including power law and negative binomial functions. This chapter has important implications for the study of transmission dynamics in general, including suggestions for experimental designs suitable for distinguishing transmission functions and the importance of accounting for the role of parasite depletion when examining transmission dynamics.

Building on this foundation of describing transmission dynamics, my third chapter examines species-specificity in the consumption of parasites by predators, and the influence of predation on transmission. Using a laboratory study based on natural communities identified through field data, I identify larval damselflies and mosquitofish as predators of *R. ondatrae* cercariae and California newt larvae as alternative hosts. In a subsequent experiment, I examine the role of both predation and alternative host mechanisms in altering transmission of *R. ondatrae* to target host amphibians. Furthermore, I necropsy amphibians and fish from natural wetlands to assess the congruence of field patterns with my laboratory study.

In chapter 4, I evaluate how parasite and predator traits and environmental conditions influence patterns of consumption to predict the consequences for transmission. I examine predation by two dominant types of aquatic predators, fish and insects, on a suite of four parasite species that varied in body size and presence in light or dark environmental conditions based on
circadian shedding patterns (Lewis et al. 1989, Combes 1991). Then, I test the importance of
predator foraging mode, predator body size, parasite size and light availability to predation on
parasites, in reference to predictions based on consumer-resource theory for aquatic systems.

A semi-realistic mesocosm experiment investigating multiple trophic interactions and
subsequent parasite transmission and host pathology, forms the basis of the fifth chapter of my
dissertation. Using damselflies as predators of parasites and dragonflies as predators of larval
amphibians, this research assesses the importance of predation to transmission dynamics by
quantifying both the direct effects of parasite consumption and the indirect effects of predators
on host behavior and morphology.

Given the critical roles that predators play in ecosystem processes it is important to
integrate their functions into disease ecology in general. My dissertation attempts to elucidate the
direct and indirect consequences of predation in disease dynamics. Through detailed laboratory
and mesocosm experiments, my research illustrates the mechanisms by which predation
influences disease, providing a foundation to develop questions addressing spatial scale and
patterns in the field. This research is extremely important in light of rapidly changing biological
communities (Pimm et al. 1995).
CHAPTER 2

BEYOND FREQUENCY AND DENSITY-DEPENDENCE: AN EXPERIMENTAL DEMONSTRATION OF THE IMPORTANCE OF NON-LINEAR TRANSMISSION DYNAMICS IN A HOST-MACROPARASITE SYSTEM

Abstract
Understanding pathogen transmission is crucial for predicting disease impacts, yet few studies test alternative functional forms of transmission and, among those that do, “classical” forms such frequency and density dependent transmission receive surprisingly little empirical support. Using interactions between amphibian hosts and trematode parasites as a model system, we used a novel experimental approach in which we varied four factors—duration of exposure, numbers of parasites, numbers of hosts, and parasite density—to differentiate among seven candidate transmission functions using a maximum likelihood approach. Our results indicated that, among the candidate models considered, non-linear forms of transmission involving either a power law or negative binomial function were the best fitting models and consistently outperformed density and frequency dependent functions. Across host-pathogen systems, non-linear functions may more accurately represent transmission dynamics thus providing more realistic predictions for infection and highlighting important potential mechanisms in addition to parasite depletion.

Introduction
Understanding the functional form of transmission has important implications for modeling disease impacts on host population dynamics, forecasting disease persistence in host populations and establishment in new populations, and understanding the evolution of virulence (McCallum et al. 2001, Fenton et al. 2002, Boots and Sasaki 2003, Holt et al. 2003, de Castro and Bolker 2005). An almost universal assumption in contemporary disease ecology is that the form of
transmission is either density or frequency dependent (Begon et al. 2002, Ryder et al. 2005, Smith et al. 2009). Density dependent transmission is characterized by a directly increasing rate of contact with population density leading to an increase in the force of infection (Begon et al. 2002). Alternatively, frequency dependent transmission assumes a constant rate of contact regardless of the host population density (Begon et al. 2002). The latter is often used to model sexually transmitted diseases and vector-borne infections, while the former has been used for most other host-pathogen models (McCallum et al. 2001).

Models using either frequency or density dependent transmission make strikingly different predictions for infection dynamics (Smith et al. 2009). Density dependent transmission predicts a threshold host population size required for pathogen persistence (Bolker and Greenfell 1995, Swinton et al. 1998). No such threshold exists under frequency dependent transmission, predicting instead that the pathogen can drive the host population to extinction (de Castro and Bolker 2005). Furthermore, the functions differ in their predictions regarding the role of community diversity in transmission of multi-host pathogens (Dobson 2004, Rudolf and Antonovics 2005). Given these important differences, using an inaccurate transmission function can lead to grossly erroneous predictions about the consequences of disease on host population dynamics (McCallum et al. 2001, Smith et al. 2009).

There is growing evidence from a variety of host-pathogen systems that density and frequency dependent transmission may fail to describe the relationship between host density and disease incidence (Morters et al. 2013). Beyond frequency and density dependence, several other functional forms have been suggested for modeling transmission, including non-linear functions (McCallum et al. 2001), though these have not been widely used. These alternative functional forms (Table 2.1) have been proposed based on observations that at low densities, contacts are
directly proportional to host and parasite density, but saturate at very high host or parasite densities, thus representing a mixture of both density and frequency-dependent properties (D’Amico et al. 1996, McCallum et al. 2001, Fenton et al. 2002). These observations highlight important parallels with models of other species interactions, including predator-prey, and host-parasitoid dynamics (McCallum et al. 2001). Indeed, density and frequency dependent transmission functions represent the extremes of the type II functional responses for predator-prey models (Antonovics et al. 1995). Mechanisms that can give rise to non-linear transmission functions include heterogeneities in the distribution of infectious particles, density dependent mortality of the pathogen, or transmission in aquatic environments (Briggs and Godfray 1995, Murray 2009). Phenomenological, non-linear transmission models can also be used to predict the outcome of host-pathogen interactions and encompass a wider range of epidemiologically relevant model behavior, including infection outbreak and collapse, or periodic coexistence of pathogen and host (Liu et al. 1986, Hochberg 1991).

To date, however, empirical studies of transmission dynamics are surprisingly rare, particularly those testing functional forms beyond frequency and density dependent transmission (Knell et al. 1996, McCallum et al. 2001, Ryder et al. 2007, Smith et al. 2009). Despite frequent use in models, including those used to manage infectious diseases, the specific form of transmission often goes untested (Knell et al. 1996, Fromont et al. 1998, Begon et al. 1999). Our literature review identified only 30 empirical tests of transmission functions in which 19 considered functional forms besides density and frequency dependent (Figure 2.1, Appendix 2A). Only three of these studies found support for either classical density and frequency dependent transmission.
The suite of transmission functions used to model transmission between the free-living infective stages (cercariae) of *Ribeiroia ondatrae* and Pacific Chorus frog (*Pseudacris regilla*) tadpoles. The form of each function used to model microparasite transmission from the literature is provided with the form used for macroparasites in this study for comparison. In the microparasite functions, $S$ is the number of susceptible individuals (analogous to hosts, $H$) and $I$ is the number of infectious individuals (analogous to cercariae, $C$). In all functions $\beta$ is the transmission parameter; assumed here to be constant in time. Additionally, $v$ is the volume of the enclosure, $p$ and $q$ are the susceptible (host) and infectious (cercariae) responses that represents how densities of each independently affect transmission efficiency, and $c$ is constant representing the ratio of unsuccessful cercariae to those successfully infecting. Finally, $k$ is the dispersion parameter for the negative binomial model.

<table>
<thead>
<tr>
<th>Transmission Form</th>
<th>Microparasite Function</th>
<th>Macroparasite Function</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Constant Risk</td>
<td>$\beta S$</td>
<td>$\beta C(t)H$</td>
<td>Rachowicz and Briggs 2007, Greer et al. 2008</td>
</tr>
<tr>
<td>Density-dependent (Mass Action)</td>
<td>$\beta S I$</td>
<td>$\beta \frac{C(t)H}{v}$</td>
<td>McCallum et al. 2001, Rachowicz and Briggs 2007</td>
</tr>
<tr>
<td>Frequency-dependent</td>
<td>$\frac{\beta S I}{N}$</td>
<td>$\beta C(t)$</td>
<td>McCallum et al. 2001, Rachowicz and Briggs 2007</td>
</tr>
<tr>
<td>Power (in I only)</td>
<td>$\beta S I^q$</td>
<td>$\beta C(t)^qH$</td>
<td>Greer et al. 2008</td>
</tr>
<tr>
<td>Asymptotic</td>
<td>$\frac{\beta S I}{c + S + I}$</td>
<td>$\frac{\beta C(t)}{c + H}$</td>
<td>Diekmann and Kretzschmar 1991, McCallum et al. 2001, Rachowicz and Briggs 2007, Greer et al. 2008</td>
</tr>
<tr>
<td>Negative Binomial</td>
<td>$k S \ln \left(1 + \frac{\beta I}{k}\right)$</td>
<td>$k \ln \left(1 + \frac{\beta C(t)}{k}\right)$</td>
<td>Briggs and Godfray 1995, McCallum et al. 2001, Rachowicz and Briggs 2007</td>
</tr>
<tr>
<td></td>
<td>$k H \ln \left(1 + \frac{\beta C(t)}{k}\right)$</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>


Figure 2.1. Results of a review of 30 empirical studies testing functional forms of transmission across pathogen types (see list of studies in Appendix 2A). Black bars indicate the number of studies that only considered frequency or density dependent functions (linear functions), while the gray bars represent the number of studies that considered both linear and non-linear models and found support for linear models. Finally, the white bars represent the number of studies out that found support for non-linear models when they were considered.
In the cases where empirical data have been used to test a variety of competing functions beside density and frequency dependent there has been support for non-linear functions (D’Amico et al. 1996, Fenton et al. 2002, Greer et al. 2008, Appendix 2A). A persistent challenge is that differentiating among alternative functional forms requires careful attention to experimental design; independent manipulation of both host and parasite numbers is often required to mathematically separate out density and frequency dependent forms, yet many studies vary only one or the other such that the data provide few insights about functional form (May and Anderson 1979, McCallum et al. 2001). It is important to develop a general framework for modeling transmission dynamics that can be extended to a broad range of pathogen life histories and ecosystems. This framework can then identify knowledge gaps and research priorities in need of system specific models and ideally be parameterized with laboratory or field data (Molnar et al. 2013).

In this study, we use an experimental approach to test a suite of mathematical functions as competing hypotheses for transmission dynamics of a model system involving interactions between amphibian hosts and trematode parasites. Macroparasite transmission involves analogous processes as microparasite transmission (i.e. bacteria and viruses) with added mechanistic control. Specifically, macroparasites infect though individual free-living stages that can be quantified in the host and represent unique transmission events. This allows us to decouple contact with hosts and the production of new infectious stages to generate a better understanding relative to microparasites. To evaluate how transmission depends on underlying factors, we use a maximum likelihood approach to determine which mathematical function of the transmission is best supported by the experimental data (Rachowicz and Briggs 2007). Given that an understanding of the mechanisms underlying transmission is critical to applying models,
we incorporated the depletion of parasites through infection, which is an important biological influence on transmission, into our models (Civitello et al. 2013). To isolate host behavioral mechanisms underlying the different forms of transmission, we also experimentally manipulated host behavior using anesthesia. Finally, we tested the generality of our conclusions by analyzing two other existing datasets for macroparasite transmission (Karvonen et al. 2003, Paller et al. 2007), ultimately finding that that non-linear forms of transmission, including power-law and negative binomial models, were more appropriate functions for modeling transmission than either frequency or density dependent functions. Our results have important implications for predicting how host-parasite interactions occur nature, particularly the role of community diversity in mediating transmission, as well as for providing a link between parasite-host interactions and predator-prey and host-parasitoid (parasitic organisms requiring obligate host mortality during their lifecycle) dynamics.

Methods

Study system

*Ribeiroia ondatrae* is a trematode with a complex life cycle, sequentially infecting snails (*Helisoma trivolvis*), larval amphibians, and finally amphibian-eating birds (Johnson et al. 2004). Transmission of *R. ondatrae* from snail to amphibians occurs through direct infection by free-living aquatic parasite stages known as cercariae, which then form encysted metacercariae in the amphibian. The metacercariae have been experimentally shown to induce developmental malformations in amphibians (Johnson et al. 1999). The pacific chorus frog, *Pseudacris regilla* serves as our focal amphibian host because it is among the species with the highest reported frequencies of malformations, approaching 90% of emerging metamorphs in natural populations.
(Johnson et al. 1999). Mortality and pathology in the amphibian are related to the intensity of infection, or the total number of parasites in the host, highlighting the need for understanding transmission dynamics.

**Model development and evaluation**

We evaluated seven candidate functions representing unique hypotheses for pathogen transmission with experimental data using a maximum likelihood approach following the methods of Rachowicz and Briggs (2007) and Greer et al. (2008). We obtained candidate functional forms (Table 2.1) through a comprehensive literature search of functions used to model diverse systems including microparasites, macroparasites, or parasitoids. These include the canonical density-dependent (mass action) and frequency dependent transmission functions as well as non-linear models such as two power law functions, asymptotic, and negative-binomial functions and constant risk (McCallum et al. 2001). We adapted each function as necessary to apply specifically to a macroparasite system of free-living infective stages (cercariae, $C$) and amphibian larvae (hosts, $H$). Because of confusion arising from the inconsistent use of terminology, we represent populations of hosts and parasites as numbers with density included explicitly, when applicable, by incorporating volume ($v$) into the functional forms (Begon et al. 2002). Importantly, we accounted for mechanisms of parasite depletion by allowing free-living parasite number to decline as a result of successful infection. This meant that all functional forms were given equal mechanistic footing. The latter is crucial for a fair comparison of transmission functional forms, since models with transmission mechanisms can vastly outperform models lacking mechanisms (Civitello et al. 2013).
For macroparasites, a single host can become infected by multiple independent cercariae, and appropriate models should thus represent conversion of free-living infective stages into individual encysted metacercariae (M) within the host (May and Anderson 1979). We modeled the rate of loss of cercariae due to infection (i.e., conversion of cercariae into metacercariae) with the ordinary differential equation \( \frac{dC}{dt} = -\phi C \), where \( C \) is the number of cercariae present at time \( t \), and \( \phi \) is the transmission function, which may take any of functional forms given in Table 2.1. For example, \( \phi = \beta H \) represents the constant risk function. We ignored any other sources of cercariae mortality over the short time frame of our experiment (15–240 minutes) given the age of the parasites (Karvonen et al. 2003, Paller et al. 2007). We assumed that the binomial distribution:

\[
P_n(t) = \binom{C(0)}{n} \exp\{-n\phi t\} [1 - \exp\{-\phi t\}]^{(C(0)-n)}
\]

described the distribution of unsuccessful cercariae at the end of the experiment, for \( n = 0, 1, \ldots, C(0) \), where \( C(0) \) is the initial number of cercariae and \( t \) (minutes) is the duration of the experiment.

For the first six transmission functions listed in Table 2.1, we found the analytical solutions to each differential equation using Mathematica (version 8.0.4.0, Wolfram Research, Inc.) and used predictions from these functions to aid the development of our laboratory experiments manipulating the various parameters (Table 2.2). For these transmission functions, we used the approach outlined in Bolker (2008) to minimize the negative log likelihood for the model given the data from each experiment on the numbers of cercariae successfully infecting as metacercariae using R (R Development Core Team 2008). For the negative binomial function, there was no analytical solution, so we solved it numerically using the deSolve package in R, using the same approach for minimizing the negative log likelihood for data from each
experiment. We verified that this method was equivalent to the method used for the other functions by analyzing the constant risk model with both methods and obtaining identical results.

There are two different formulations for the negative binomial model—one with hosts and one without—used in the literature (Briggs and Godfray 1995, Rachowicz and Briggs 2007, Greer et al. 2008). Under three of our experimental manipulations where hosts equal one the two functions make identical predictions; however, for the host density experiment (described below), we evaluated both versions. We used corrected Akaike information criterion (AICc) values and Akaike weights (interpreted as the probability that the model is the best among the candidate models) to compare the different transmission functions (Burnham and Anderson 2002, Greer et al. 2008).
Table 2.2. Equations representing the analytical solutions for the corresponding transmission functions tested with empirical data. The variable column represents the predictions of each model with respect to the particular variable. All models predict an increase in transmission with increases in the initial numbers of parasites ($N_p$). Increasing the number of hosts (H) can have positive, negative, or no effect on transmission. Volume ($v$) is only used in the function for density dependent transmission and has a negative influence on transmission.

<table>
<thead>
<tr>
<th>Transmission Form</th>
<th>Equation</th>
<th>Variable</th>
</tr>
</thead>
<tbody>
<tr>
<td>Constant Risk</td>
<td>$M(t) = N_p (1 - e^{-\beta H t})$</td>
<td>$N_p$ $+$ $H$ $+$ $v$ NA</td>
</tr>
<tr>
<td>Density-dependent (Mass Action)</td>
<td>$M(t) = N_p \left(1 - e^{-\frac{\beta t H}{v}}\right)$</td>
<td>$+$ $+$ $-$</td>
</tr>
<tr>
<td>Frequency-dependent</td>
<td>$M(t) = N_p (1 - e^{-\beta t})$</td>
<td>$+$ NA NA</td>
</tr>
<tr>
<td>Power (in I only)</td>
<td>$M(t) = N_p - (N_p^{1-q} + \beta (-1 + q)tH)^{\frac{1}{1-q}}$</td>
<td>$+$ $+$ NA</td>
</tr>
<tr>
<td>Power (in S and I)</td>
<td>$M(t) = N_p - (N_p^{1-q} + \beta (-1 + q)tH^p)^{\frac{1}{1-q}}$</td>
<td>$+$ $+$ NA</td>
</tr>
<tr>
<td>Asymptotic</td>
<td>$M(t) = N_p \left(1 - e^{\frac{-\beta t}{v^2}}\right)$</td>
<td>$+$ $-$ NA</td>
</tr>
</tbody>
</table>
Laboratory transmission experiments

To test our seven hypotheses for transmission dynamics (Table 2.1), we used a series of laboratory experiments that manipulated different variables independently that caused the models to make divergent predictions. We used *P. regilla* tadpoles raised in the laboratory from eggs and *R. ondatrae* from naturally infected snails collected from the field (Appendix 2B). While other studies have investigated transmission with respect to system specific mechanism, such as age of hosts or feeding behavior (Goulson et al. 1995, D’Amico et al. 1996), we sought to manipulate factors relevant across host-pathogen systems and modes of transmission. Several previous studies have examined subsets of these factors in isolation or in combination (Anderson and May 1979, D’Amico et al. 1996, Knell et al. 1996, Karvonen et al. 2003, Paller et al. 2007). However, in some cases densities and numbers of hosts were confounded. Furthermore, as we show below, one of the most common types of experiments does not reveal any distinction between transmission functions (see also Antonovics and Alexander 1992, Rachowics and Briggs 2007).

Specifically, we conducted four independent experiments in which we varied (1) parasite number (4, 13, 30, 63, & 144 parasites), (2) host density (0.48, 0.95, & 1.9 tadpoles/L), (3) duration of exposure (15, 30, 60, 120, & 240 minutes), and (4) parasite density (5, 15, 30, 45 & 60 parasites/L), where each treatment level was replicated ten times. Parasite number was manipulated independently of parasite density by varying volume while maintaining the same parasite density (30 parasites/L, range of volumes 0.12-4.8L). Host density may result in crowding which may have other additional effects on host susceptibility due to stress or resource limitation; however, our trials lasted only 30 minutes so we assumed that these effects would be minor (Rachowicz and Briggs 2007). Finally, our experimental design allowed us to minimize many of the mechanisms that could potentially drive non-linear transmission dynamics,
including heterogeneity in hosts and parasites by using tadpoles that were carefully matched to size and developmental stage and cercariae that were collected within a narrow age range (Appendix 2C). We also used a short duration of exposure and small container sizes that reduced any effect of spatial heterogeneity.

**Host behavior experiment**

To examine the role of host behavior in influencing the form of transmission, we performed an additional experiment where we reduced host activity by anesthesia with neutral buffered MS-222 (0.125% tricaine methanesulfonate for 3 minutes, Appendix 2D). By anesthetizing tadpoles for the duration of the exposure period, we eliminated effects of anti-parasite behavior, including evasive maneuvers (Daly and Johnson 2011). We followed the same procedures used for varying parasite number while maintaining parasite density across container volumes (see above). We had 4 to 7 replicates per container volume (parasite number) due 12 tadpoles that recovered from anesthesia before the end of the exposure time or died and were excluded from analysis. After each experimental procedure, we maintained tadpoles for 48 hours before quantifying parasite infection (Appendix 2E). In addition to model fitting to test our transmission hypotheses, we also examined the role of host activity influencing overall infection intensity as proportion of cercariae that successfully infected a host using generalized linear models with number of cercariae exposed and anesthetized or unanesthetized as treatment effects and quasibinomial errors (Crawley 2007, R Development Core Team 2008).
Evaluation of other host-macroparasite systems

We tested the generality of our findings with *R. ondatrae* and amphibian hosts by extending our analysis to previously collected data from two additional macroparasite systems: rainbow trout (*Oncorhynchus mykiss*) and *Diplostomum spathaceum* (Karvonen et al. 2003) and minnow (*Zacco temmincki*) and *Centrocestus armatus* (Paller et al. 2007, Appendix 2F). These studies used experimental procedures where individual hosts were exposed to infective cercariae at (i) different densities and (ii) numbers independently of density. However, unlike our experiments, they did not manipulate host density or duration of exposure. Finally, they evaluated only frequency and density dependent functions.

Results

Laboratory transmission experiments

1. Varying parasite number (constant parasite density, variable volume)

In the experiment where we varied the number of parasites while keeping parasite density constant, we observed a non-linear saturation of infection with increasing exposure (Figure 2.2A). This relationship was best represented by the negative binomial function (Akaike Weight = 0.99). Density dependent transmission had the weakest support (Δ AICc 1088.76) due to the strong negative relationship it predicted with the simultaneous decline in host density (Figure 2.
Figure 2.2. The number of *Ribeiroia ondatrae* metacercariae infecting *Pseudacris regilla* tadpoles in laboratory experiments manipulating A) Parasite Number, B) Host Number, C) Duration of Exposure, D) Parasite Density. Points in A, C-D represent the infection levels of individual tadpoles, while B represents the total infection level of all tadpoles in a given density treatment. Lines represent the average infection expected from the seven transmission functions with their best-fit parameters color-coded by fit (Table 2.3: black = most support, blue, red, green = least support). If models made identical predictions lines were combined for clarity. Note different scales of the x and y axes.
2A). Linear models, such as frequency dependent transmission underestimated transmission success at the low exposure levels and overestimated infection at the high exposures (Figure 2.2A). Overall, there was a sharp decline in the proportion of successful cercariae from 0.7 to 0.1 over the range of container volumes tested (0.12 – 4.8L).

2. Varying host density (constant volume, variable host number)

We observed a slightly non-linear increase in total cercariae transmission success with increasing host density from 20 percent with a single host to 53 percent with four tadpoles. Based on this relationship, we could clearly rule out functions that did not include a host component, such as frequency dependent, asymptotic, or negative binomial (Δ AICc 144-147; Figure 2.2B). When we evaluated the second version of the negative binomial function, which included hosts as a variable, the fit was substantially improved but it was still not among the best fitting models (Δ AICc = 6). The function with the highest support was power law 1, with an exponent of 1.8 on the cercariae variable (Akaike Weight = 0.66). The best fitting parameter values for the power law 2 function exponents were both close to 1, in which case the function simplifies to both constant risk and density dependent transmission when volume is constant (3-4 Δ AICc).

3. Varying duration of exposure

When we manipulated the duration of exposure, we found a rapid increase in the proportion of successful cercariae between 15 and 60 minutes from 0.3 to over 0.7 followed by saturation of infection at ~ 0.8 over the longest exposure periods (Figure 2.2C). Based on this non-linear saturation, we identified both power law functions (power law 1, Akaike Weight = 0.76; power law 2, Akaike Weight = 0.24) as the best fitting models. All remaining functions underestimated
the rapid transmission dynamics initially and overestimated the total infection at saturation (Δ AICc 184-186).

4. **Varying parasite density (constant volume, variable parasite number)**

Finally, we observed a linear increase in average infection from ~1 to 14 cercariae with increasing parasite density (Figure 2.2D). This experimental manipulation provided the least ability to differentiate among the competing transmission functions. We found almost equal support across all the tested functions (Δ AICc 1-3). Interestingly, proportion of successful cercariae remained relatively constant over the range of densities from ~0.17 to 0.28.

**Summary of Manipulations of Four Factors**

The three experimental manipulations that provided the best distinction between the competing functions were varying the number of parasites independently of density, host density, and duration of exposure (Figure 2.2). Across most experiments, the maximum likelihood analysis strongly supported two types of non-linear functions, namely the power law and negative binomial functions, while classical linear models of frequency or density dependent were not well supported (Table 2.3). Importantly, the negative binomial did not provide an adequate fit to the data when we varied host density. Therefore, we find the strongest support for the hypothesis represented by the power law 1 function, indicating that allowing transmission to scale non-linearly with respect to numbers of cercariae is important for accurately capturing transmission dynamics.
Table 2.3. Maximum likelihood fitting of transmission functions to data from laboratory experiment of *Ribeiroia ondatrae* cercariae transmission to *Pseudacris regilla* tadpoles evaluated according to AICc.

<table>
<thead>
<tr>
<th>Transmission Function</th>
<th>β (units)</th>
<th>Additional Parameters (units)</th>
<th>AICc value</th>
<th>Δ AICc</th>
<th>Akaike Weight</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>1. Varying parasite number (constant parasite density, variable volume)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Constant Risk (CR)</td>
<td>0.441 (min⁻¹)</td>
<td></td>
<td>539.27</td>
<td>130.52</td>
<td>0.00</td>
</tr>
<tr>
<td>Density Dependent (Den)</td>
<td>0.551 (H⁻¹ min⁻¹)</td>
<td></td>
<td>1497.51</td>
<td>1088.76</td>
<td>0.00</td>
</tr>
<tr>
<td>Frequency Dependent (Freq)</td>
<td>0.441 (min⁻¹)</td>
<td>q = 0.41 (dimension-less)</td>
<td>637.84</td>
<td>229.09</td>
<td>0.00</td>
</tr>
<tr>
<td>Power I (Pow 1)</td>
<td>4.860 (H⁻¹ p⁻q min⁻¹)</td>
<td>p = -3.29, q = 0.41 (dimension-less)</td>
<td>418.65</td>
<td>9.90</td>
<td>0.01</td>
</tr>
<tr>
<td>Power SI (Pow 2)</td>
<td>0.290 (min⁻¹)</td>
<td>c = -0.34 (H)</td>
<td>640.01</td>
<td>231.27</td>
<td>0.00</td>
</tr>
<tr>
<td>Asymptotic (Asympt) Negative Binomial (NB)</td>
<td>3.861 (min⁻¹)</td>
<td>k = 8.07 (min⁻¹)</td>
<td>408.74</td>
<td>0.00</td>
<td>0.99</td>
</tr>
<tr>
<td><strong>2. Varying host density (constant volume, variable host number)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Constant Risk (CR)</td>
<td>0.424 (min⁻¹)</td>
<td></td>
<td>229.62</td>
<td>3.84</td>
<td>0.10</td>
</tr>
<tr>
<td>Density Dependent (Den)</td>
<td>1.869 (H⁻¹ min⁻¹)</td>
<td></td>
<td>229.62</td>
<td>3.84</td>
<td>0.10</td>
</tr>
<tr>
<td>Frequency Dependent (Freq)</td>
<td>0.892 (min⁻¹)</td>
<td>q = 1.82  (dimension-less)</td>
<td>370.49</td>
<td>144.71</td>
<td>0.00</td>
</tr>
<tr>
<td>Power I (Pow 1)</td>
<td>0.018 (H⁻¹ p⁻q min⁻¹)</td>
<td>p = 0.90 , q = 1.33 (dimension-less)</td>
<td>225.78</td>
<td>0.00</td>
<td>0.66</td>
</tr>
<tr>
<td>Power SI (Pow 2)</td>
<td>0.128 (H⁻¹ p⁻q min⁻¹)</td>
<td></td>
<td>228.82</td>
<td>3.04</td>
<td>0.14</td>
</tr>
<tr>
<td>Asymptotic (Asympt) Negative Binomial (NB)</td>
<td>6072735 (min⁻¹)</td>
<td>c = 6807703 (H)</td>
<td>372.79</td>
<td>147.01</td>
<td>0.00</td>
</tr>
<tr>
<td>Negative Binomial Host (NBH)</td>
<td>13.142 (min⁻¹)</td>
<td>k = 10.98 (min⁻¹)</td>
<td>372.79</td>
<td>147.01</td>
<td>0.00</td>
</tr>
<tr>
<td></td>
<td>0.424 (H⁻¹ min⁻¹)</td>
<td>k = 637705.8 (min⁻¹)</td>
<td>231.93</td>
<td>6.14</td>
<td>0.03</td>
</tr>
<tr>
<td><strong>3. Varying duration of exposure</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Constant Risk (CR)</td>
<td>0.013 (min⁻¹)</td>
<td></td>
<td>485.10</td>
<td>183.53</td>
<td>0.00</td>
</tr>
<tr>
<td>Density Dependent (Den)</td>
<td>0.013 (H⁻¹ min⁻¹)</td>
<td></td>
<td>485.10</td>
<td>183.53</td>
<td>0.00</td>
</tr>
<tr>
<td>Frequency Dependent (Freq)</td>
<td>0.013 (min⁻¹)</td>
<td></td>
<td>485.10</td>
<td>183.53</td>
<td>0.00</td>
</tr>
</tbody>
</table>
### Power I (Pow 1)
- $0.003 \left( H^q \text{min}^{-1} \right)$
- $q = 2.39$ (dimension-less)
- $301.57$ 
- $0.00$ 
- $0.76$

### Power SI (Pow 2)
- $0.003 \left( H^{1-p} \text{min}^{-1} \right)$
- $p = -2.96 \; , \; q = 2.39$ (dimension-less)
- $303.84$ 
- $2.27$ 
- $0.24$

### Asymptotic (Asympt)
- $0.013 \left( \text{min}^{-1} \right)$
- $c = -0.013 \left( H \right)$
- $487.27$ 
- $185.70$ 
- $0.00$

### Negative Binomial (NB)
- $0.013 \left( \text{min}^{-1} \right)$
- $k = 402034.2 \left( \text{min}^{-1} \right)$
- $487.27$ 
- $185.70$ 
- $0.00$

### Constant Risk (CR)
- $0.482 \left( \text{min}^{-1} \right)$
- $372.33$ 
- $0.00$ 
- $0.22$

### Density Dependent (Den)
- $0.482 \left( H^1 \text{min}^{-1} \right)$
- $372.33$ 
- $0.00$ 
- $0.22$

### Frequency Dependent (Freq)
- $0.482 \left( \text{min}^{-1} \right)$
- $372.33$ 
- $0.00$ 
- $0.22$

### Power I (Pow 1)
- $0.681 \left( H^q \text{min}^{-1} \right)$
- $q = 0.90$ (dimension-less)
- $373.43$ 
- $1.10$ 
- $0.13$

### Power SI (Pow 2)
- $0.681 \left( H^{1-p} \text{min}^{-1} \right)$
- $p = 0.10 \; , \; q = 0.90$ (dimension-less)
- $375.70$ 
- $3.36$ 
- $0.04$

### Asymptotic (Asympt)
- $0.307 \left( \text{min}^{-1} \right)$
- $c = -0.36 \left( H \right)$
- $374.51$ 
- $2.17$ 
- $0.08$

### Negative Binomial (NB)
- $0.529 \left( \text{min}^{-1} \right)$
- $k = 101.97 \left( \text{min}^{-1} \right)$
- $374.20$ 
- $1.87$ 
- $0.09$

### Host behavior experiment

#### Constant Risk (CR)
- $0.789 \left( \text{min}^{-1} \right)$
- $373.99$ 
- $168.13$ 
- $0.00$

#### Density Dependent (Den)
- $1.078 \left( H^1 \text{min}^{-1} \right)$
- $701.48$ 
- $495.61$ 
- $0.00$

#### Frequency Dependent (Freq)
- $0.789 \left( \text{min}^{-1} \right)$
- $373.99$ 
- $168.13$ 
- $0.00$

#### Power I (Pow 1)
- $6.788 \left( H^q \text{min}^{-1} \right)$
- $q = 0.45$ (dimension-less)
- $217.68$ 
- $11.82$ 
- $0.00$

#### Power SI (Pow 2)
- $6.792 \left( H^{1-p} \text{min}^{-1} \right)$
- $p = -2.61 \; , \; q = 0.45$ (dimension-less)
- $220.28$ 
- $14.42$ 
- $0.00$

#### Asymptotic (Asympt)
- $0.409 \left( \text{min}^{-1} \right)$
- $c = -0.48 \left( H \right)$
- $376.37$ 
- $170.50$ 
- $0.00$

#### Negative Binomial (NB)
- $4.770 \left( \text{min}^{-1} \right)$
- $k = 14.69 \left( \text{min}^{-1} \right)$
- $205.86$ 
- $0.00$ 
- $1.00$
Host behavior experiment

Our anesthesia procedure was successful in reducing tadpole activity including potential evasive, anti-parasite behaviors, with hosts remaining motionless over the 30-minute experimental period. Overall, we observed an average 46 percent higher infection success in the experiment where we anesthetized tadpoles compared to the same experimental design with unmanipulated tadpoles (GLM, anesthesia treatment $Z = 2.2, p = 0.031$). Intriguingly, we observed a non-linear, saturating relationship with infection increasing rapidly from ~4 to 19 and then leveling off at an average of 25 metacercariae across the range of parasite exposures. This was best represented by the negative binomial function (Akaike Weight = 1.0; Figure 2.3), the matching functional form supported by the same experimental manipulation of parasite numbers with constant density with unmanipulated hosts (Figure 2.2A). This experiment shows that host anti-parasite behavior matters for net transmission, but is unlikely to be responsible for the non-linear dynamics observed per se.

Evaluation of other host-macroparasite systems

Our analysis of data from previous studies of macroparasite transmission showed congruence with our empirical results, suggesting that non-linear transmission functions are general across a variety of parasite and host taxa and different scales of experimental procedures (Table 2.4). Importantly, the results for both systems stand in contrast to the original conclusions that the transmission mode was frequency dependent (Karvonen et al. 2003, Paller et al. 2007, Figure 2.4). However, Karvonen et al. (2003) log transformed the data prior to analysis, which may have lead to the support for a linear function for otherwise non-linear dynamics. Similar to our
empirical data, experiments manipulating parasite density were less able to distinguish between functional forms compared to parasite numbers varied independently of density.

For the case of rainbow trout (*Oncorhynchus mykiss*) and *Diplostomum spathaceum* cercariae (Karvonen et al. 2003), we observed a rapid increase in transmission followed by saturation at the highest exposure levels when the number of parasites was varied while parasite density remained constant. This supported the power law 1 model (Akaike Weight = 0.45, Figure 2.4A), which differed slightly from our version of the experiment, where we found the negative binomial function was the best fit (Figure 2.2A). For the experiment where parasite density was varied, the negative binomial and power law models were almost equally supported (Δ AICc 0.3-3); however, the trends appeared nearly linear (Figure 2.4B).

In the case of minnows (*Zacco temmincki*) and *Centrocestus armatus* cercariae (Paller et al. 2007), we also observed that the negative binomial model was most strongly supported (Akaike Weight = 0.51) when parasite numbers were varied independent of parasite density (Figure 2.4C), again capturing the apparent curvilinear relationship. Finally, for the experiment varying parasite density, we observed an almost linear relationship, but still identified the negative binomial model as the best fit to the data (Akaike Weight = 0.51) with some limited support for constant risk, density dependent, power law I, and frequency dependent functions (Δ AICc 3-4; Figure 2.4D).
Table 2.4. Maximum likelihood fitting of transmission functions to data from previous laboratory experiments (Karvonen et al. 2003, Paller et al. 2007) examining cercariae transmission to fish hosts evaluated according to AIC<sub>c</sub>.

<table>
<thead>
<tr>
<th>Transmission Function</th>
<th>β (units)</th>
<th>Additional Parameters (units)</th>
<th>AIC&lt;sub&gt;c&lt;/sub&gt; value</th>
<th>Δ AIC&lt;sub&gt;c&lt;/sub&gt;</th>
<th>Akaike Weight</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Parasite Density Karvonen et al. 2003</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Constant Risk (CR) Density Dependent (Den)</td>
<td>0.826 (min&lt;sup&gt;-1&lt;/sup&gt;) 4.82 (H&lt;sup&gt;1&lt;/sup&gt; min&lt;sup&gt;-1&lt;/sup&gt;)</td>
<td></td>
<td>299</td>
<td>5.9</td>
<td>0.02</td>
</tr>
<tr>
<td>Frequency Dependent (Freq)</td>
<td>0.826 (min&lt;sup&gt;-1&lt;/sup&gt;) 1.648 (H&lt;sup&gt;q&lt;/sup&gt; min&lt;sup&gt;-1&lt;/sup&gt;)</td>
<td>q = 0.85 (dimension-less)</td>
<td>293</td>
<td>0.4</td>
<td>0.37</td>
</tr>
<tr>
<td>Power I (Pow 1)</td>
<td>1.649 (H&lt;sup&gt;1-p-q&lt;/sup&gt; min&lt;sup&gt;-1&lt;/sup&gt;)</td>
<td>p = -0.75 , q = 0.85 (dimension-less)</td>
<td>296</td>
<td>3.0</td>
<td>0.10</td>
</tr>
<tr>
<td>Power SI (Pow 2) Asymptotic (Asympt) Negative Binomial (NB)</td>
<td>0.418 (min&lt;sup&gt;-1&lt;/sup&gt;)</td>
<td>c = -0.49 (H)</td>
<td>301</td>
<td>8.3</td>
<td>0.01</td>
</tr>
<tr>
<td></td>
<td>1.061 (min&lt;sup&gt;-1&lt;/sup&gt;)</td>
<td>k = 198.43 (min&lt;sup&gt;-1&lt;/sup&gt;)</td>
<td>293</td>
<td>0.0</td>
<td>0.45</td>
</tr>
<tr>
<td><strong>Parasite Number Karvonen et al. 2003</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Constant Risk (CR) Density Dependent (Den)</td>
<td>0.020 (min&lt;sup&gt;-1&lt;/sup&gt;) 3.852 (H&lt;sup&gt;1&lt;/sup&gt; min&lt;sup&gt;-1&lt;/sup&gt;)</td>
<td></td>
<td>2191</td>
<td>1894.4</td>
<td>0.00</td>
</tr>
<tr>
<td>Frequency Dependent (Freq)</td>
<td>0.020 (min&lt;sup&gt;-1&lt;/sup&gt;) 13.130 (H&lt;sup&gt;q&lt;/sup&gt; min&lt;sup&gt;-1&lt;/sup&gt;)</td>
<td>q = 0.26 (dimension-less)</td>
<td>297</td>
<td>0.0</td>
<td>0.45</td>
</tr>
<tr>
<td>Power I (Pow 1)</td>
<td>13.125 (H&lt;sup&gt;1-p-q&lt;/sup&gt; min&lt;sup&gt;-1&lt;/sup&gt;)</td>
<td>p = 17.63 , q = 0.26 (dimension-less)</td>
<td>299</td>
<td>2.5</td>
<td>0.13</td>
</tr>
<tr>
<td>Power SI (Pow 2) Asymptotic (Asympt) Negative Binomial (NB)</td>
<td>0.020 (min&lt;sup&gt;-1&lt;/sup&gt;)</td>
<td>c = -0.02 (H)</td>
<td>2194</td>
<td>1896.7</td>
<td>0.00</td>
</tr>
<tr>
<td></td>
<td>1.430 (min&lt;sup&gt;-1&lt;/sup&gt;)</td>
<td>k = 22.14 (min&lt;sup&gt;-1&lt;/sup&gt;)</td>
<td>318</td>
<td>20.6</td>
<td>0.00</td>
</tr>
<tr>
<td><strong>Parasite Density Paller et al. 2007</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Constant Risk (CR) Density Dependent (Den)</td>
<td>1.541 (min&lt;sup&gt;-1&lt;/sup&gt;) 0.247 (H&lt;sup&gt;1&lt;/sup&gt; min&lt;sup&gt;-1&lt;/sup&gt;)</td>
<td></td>
<td>564</td>
<td>2.9</td>
<td>0.12</td>
</tr>
<tr>
<td>Frequency Dependent (Freq)</td>
<td>1.541 (min&lt;sup&gt;-1&lt;/sup&gt;) 1.792 (H&lt;sup&gt;q&lt;/sup&gt; min&lt;sup&gt;-1&lt;/sup&gt;)</td>
<td>q = 0.97 (dimension-less)</td>
<td>564</td>
<td>2.9</td>
<td>0.12</td>
</tr>
<tr>
<td>Power I (Pow 1)</td>
<td></td>
<td></td>
<td>565</td>
<td>3.8</td>
<td>0.08</td>
</tr>
<tr>
<td>Model</td>
<td>Parameter</td>
<td>Value</td>
<td>N</td>
<td>Mean</td>
<td>SD</td>
</tr>
<tr>
<td>----------------------------</td>
<td>-----------</td>
<td>--------------------</td>
<td>----</td>
<td>------</td>
<td>-----</td>
</tr>
<tr>
<td>Power SI (Pow 2)</td>
<td>p</td>
<td>-3.41, q = 0.97</td>
<td>567</td>
<td>6.4</td>
<td>0.02</td>
</tr>
<tr>
<td>Asymptotic (Asympt)</td>
<td>c</td>
<td>-0.65 (H)</td>
<td>566</td>
<td>5.3</td>
<td>0.04</td>
</tr>
<tr>
<td>Negative Binomial (NB)</td>
<td>k</td>
<td>1591.16 (min⁻¹)</td>
<td>561</td>
<td>0.0</td>
<td>0.51</td>
</tr>
</tbody>
</table>

**Parasite Number Paller et al. 2007**

<table>
<thead>
<tr>
<th>Model</th>
<th>Parameter</th>
<th>Value</th>
<th>N</th>
<th>Mean</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Constant Risk (CR)</td>
<td></td>
<td>0.616 (min⁻¹)</td>
<td>1886</td>
<td>921.7</td>
<td>0.00</td>
</tr>
<tr>
<td>Density Dependent (Den)</td>
<td></td>
<td>0.327 (H⁻¹ min⁻¹)</td>
<td>11542</td>
<td>10577.9</td>
<td>0.00</td>
</tr>
<tr>
<td>Frequency Dependent (Freq)</td>
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<td>0.616 (min⁻¹)</td>
<td>1886</td>
<td>921.7</td>
<td>0.00</td>
</tr>
<tr>
<td>Power I (Pow 1)</td>
<td></td>
<td>6.931 (H⁻¹ min⁻¹)</td>
<td>1055</td>
<td>90.7</td>
<td>0.00</td>
</tr>
<tr>
<td>Power SI (Pow 2)</td>
<td>p</td>
<td>-13.74, q = 0.60</td>
<td>1057</td>
<td>93.1</td>
<td>0.00</td>
</tr>
<tr>
<td>Asymptotic (Asympt)</td>
<td>c</td>
<td>-0.42 (H)</td>
<td>1888</td>
<td>923.9</td>
<td>0.00</td>
</tr>
<tr>
<td>Negative Binomial (NB)</td>
<td>k</td>
<td>139.94 (min⁻¹)</td>
<td>964</td>
<td>0.0</td>
<td>0.51</td>
</tr>
</tbody>
</table>
Figure 2.3. The number of *Ribeiroia ondatrae* metacercariae infecting *Pseudacris regilla* tadpoles in laboratory experiments manipulating host behavior under conditions varying parasite number (see text). Points represent the infection levels of individual tadpoles. Lines represent the average infection expected from the seven transmission functions with their best-fit parameters color-coded by fit (Table 2.3: black = most support, blue, red, green = least support). If models made identical predictions lines were combined for clarity.
Figure 2.4. The number of *Diplostomum spathaceum* metacercariae infecting rainbow trout (*Oncorhynchus mykiss*) across a range of A. parasite numbers and B. parasite density (data from Karvonen et al. 2003). The number of *Centrocestus armatus* metacercariae attached to minnow (*Zacco temmincki*) across a range of C. parasite numbers and D. parasite density (data from Paller et al. 2007). Points represent the infection levels of individual fish. Lines represent the average infection expected from the seven transmission functions with their best-fit parameters color-coded by fit (Table 2.4: black = most support, blue, red, green = least support). If models made identical predictions lines were combined for clarity. Note different scales of the x and y axes.
Discussion

By using an array of experimental manipulations, we demonstrate that transmission of *R. ondatrae* cercariae scales in a positive but non-linear fashion with the number of cercariae, number of hosts, and duration of exposure. As suggested by recent evaluations (Civitello et al. 2013), the transmission functions we evaluated all incorporated one of the key mechanisms affecting dynamics in many macroparasite systems: decreasing parasite number/density as infections occur. However, even with this biological realism, these functional forms remain general enough to be applicable to other disease systems.

Comparing the performance of this wide variety of functional forms across four different experiments gave us greater power than previous studies to evaluate alternative hypotheses about transmission. This led to insights in two important areas. First, parasite density has been the most commonly manipulated variable in previous studies of transmission; yet our results, including our analysis of previously published data, show that this is the least useful experiment for distinguishing among transmission forms. We were additionally able to identify experimental approaches more appropriate for distinguishing among competing models (varying parasite numbers while controlling for parasite density, host density, and duration of exposure). In other words, our results will aid future investigators seeking to design experiments that offer the maximum power for discriminating among alternative hypotheses about transmission.

Second, although modeling transmission plays a key role in forecasting consequences of disease, there is still a lack of empirical study and a generalized framework for macroparasites. Our study provides a critical empirical test of transmission functions, filling important knowledge gaps for vertebrate pathogens and parasites with free-living infective stages (Rachowicz and Briggs 2007). Often parasites with free-living infective stages are assumed to
have the same transmission mode as directly transmitted pathogens or they are not explicitly modeled because of the short time scale of the dynamics (May and Anderson 1979, Rachowicz and Briggs 2007). Our results show that there are non-linear patterns of saturation with different experimental conditions, indicating that prior assumptions of classical transmission functions may be an oversimplification of important underlying dynamics. Previous studies of macroparasites have investigated limited transmission functions and concluded that transmission was frequency dependent. However, our re-analysis found that non-linear forms of transmission were a superior fit to their experimental data. By transforming their data prior to analysis, non-linear patterns in transmission may have been obscured (Karvonen et al. 2003, Paller et al. 2007, i.e., linear fits to log-transformed data usually indicate power-law relationships). Among the macroparasites of humans, *Schistosoma* sp. are arguably the most important, but key facets of their transmission dynamics have received relatively little empirical study (Anderson 1978, Carter 1982). Therefore, functional forms have been applied based to address questions about control with little empirical basis for those choices (Woolhouse 1992). Our experimental design and candidate modeling approach, coupled with the traits of macroparasites that make them an ideal model system, should encourage future investigations of additional systems, particularly for human pathogens.

Unified, general models of the transmission process are essential for comparison of dynamic processes in different systems and for studies of the evolution of the transmission process itself (Antonovics et al. 1995, Fenton et al. 2002). Non-linear transmission functions appear to be relevant across a variety of host-pathogen systems, providing a possible general framework for understanding transmission dynamics (Appendix 2A). Non-linear models have been used based on characteristics of the host-pathogen interactions or because they are more
flexible than classic models, even without empirical tests. Some examples include modeling insect pathogens (Briggs and Godfray 1995), chytrid infection in natural populations of *Daphnia* (Johnson et al. 2009), and *Schistosoma* sp. cercariae (Gao et al. 2011). However, it is difficult to compare across studies because of the diversity of non-linear functional forms examined, some of which are specific to only one or a small subset of systems because they are based on traits such as feeding behavior or age structure (Goulson et al. 1995, Knell et al. 1996, Dwyer et al. 2000, Civitello et al. 2013). It has also been suggested that non-linear transmission modes are general to aquatic diseases (Murray 2009). Indeed, there was some evidence to suggest that transmission of *Batrachochytrium dendrobatidis* and *Ambystoma tigrinum* virus (ATV) through aquatic environments may be non-linear (Rachowicz and Briggs 2007, Greer et al. 2008). However, most previous studies of transmission are of directly transmitted diseases in terrestrial environments leaving few aquatic systems for direct comparison (McCallum et al. 2004, Murray 2009). Accordingly, more empirical tests of multiple competing transmission functions are needed, especially where (1) density or frequency dependent transmission have been used previously and/or (2) parasite density, the least informative manipulation, is the only manipulation that has been performed.

Identifying the specific mechanisms responsible for transmission dynamics has recently been highlighted as a priority for disease research (e.g., Civitello et al. 2013). Multiple mechanisms resulting in non-linear transmission dynamics have been previously proposed, including aggregation in the distribution of hosts or parasites in the environment, heterogeneity in host susceptibility (immunity and physiology), and behavior of hosts or parasitoids (Hochberg 1991, Briggs and Godfray 1995, Murray 2009). Since depletion of parasites, whether by host foraging ecology or direct infection, has been identified as a key mechanism in systems like ours
(Civitello et al. 2013), we incorporated parasite depletion in all the phenomenological models we used. Our experimental design allowed us to further eliminate or minimize the role of other mechanisms. The short time scale of the experiment eliminated heterogeneity in the mortality of cercariae. Importantly, we also performed treatments that eliminated effects of anti-parasite behavior on transmission by anesthetizing hosts. This indicated that anti-parasite behavior was not necessary for the non-linear patterns observed.

By considering other ecological interactions represented by non-linear functions, we can integrate transmission dynamics with concepts from consumer resource dynamics and natural enemy ecology. In our models, the transmission coefficient is analogous to the searching efficiency of parasitoid (Knell et al. 1996). Transmission functions are also analogous to the functional response of predators (McCallum et al. 2001). For example, saturating functions for transmission are similar to the Holling type II functional response (Antonovics et al. 1995, McCallum et al. 2001). This suggests that cercariae behavior might be similar to parasitoids or predators and lends support for further investigations on the role of cercariae behavior in transmission (Combes et al. 2002).

Understanding transmission dynamics has important implications for disease management and conservation. Because of their flexibility, non-linear forms of transmission may be more useful in forecasting disease as opposed to strictly frequency or density dependent transmission when the true functional form for transmission is unknown. Non-linear functions lead to complex dynamics in terms of stability and the thresholds for pathogen invasion in host populations (Liu et al. 1986, Hochberg 1991). Most importantly, non-linear terms suggest that there is also a threshold density of infected hosts or infective stages required for the disease to persist, rather than only a threshold density of susceptibles (Gubbins and Gilligan 1997). If an
inappropriate transmission function is used it could lead to erroneous conclusions about the risk of pathogen regulation or induced extinction of host populations or effectiveness of control strategies or the role of biodiversity in regulating transmission (Hochberg 1989, Dobson 2004, de Castro and Bolker 2005, Rudolf and Antonovics 2005, Morters et al. 2013). Our experiments may accurately describe transmission on an individual level, but whether these functions apply to transmission at a population level requires future research at more natural spatial scales (Dwyer and Elkinton 1993, Goulson et al. 1995). In addition, it will be important to place non-linear transmission functions into a full modeling framework incorporating population dynamics of the hosts to test the impacts on infection, patterns of aggregation, and ultimately population dynamics of hosts and parasites (Fromont et al. 1998).

Appendices

Appendix 2A. Review of empirical forms of transmission

We searched Web of Science and citations in relevant papers to identify empirical tests of transmission functions. We used search terms (transmission*, disease*, pathogen*, model*, empirical*, test*) to identify potential papers for inclusion. We established criteria for inclusion in our review. First, papers had to manipulate variables involved in transmission dynamics and evaluate competing functional forms of transmission beyond simply fitting a single functional form. Based on these criteria we identified 30 studies for our review and the citations and extracted data are provided in Table 2.5.
Table 2.5 List of studies and extracted data for literature review on the empirical tests of pathogen transmission functions.

<table>
<thead>
<tr>
<th>Type of Pathogen</th>
<th>Pathogen</th>
<th>Host</th>
<th>Transmission Function</th>
<th>Method</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacterium</td>
<td><em>Tuberculosis</em> (Mycobacterium <em>bovis</em>)</td>
<td>Possum (Trichosurus <em>vulpecula</em>)</td>
<td>Negative Binomial</td>
<td>Simulation</td>
<td>1</td>
</tr>
<tr>
<td>Bacterium</td>
<td><em>Pasteurir ramosa</em></td>
<td><em>Daphnia magn</em></td>
<td>Non-linear (Both Versions)</td>
<td>Model fitting</td>
<td>2</td>
</tr>
<tr>
<td>Bacterium</td>
<td><em>Leptospira interrogans</em></td>
<td>Brushtail possums (Trichosurus <em>vulpecula</em>)</td>
<td>Density Dependent</td>
<td>Model fitting</td>
<td>3</td>
</tr>
<tr>
<td>Bacterium</td>
<td>Brucella</td>
<td>Bison (<em>Bison bison</em>)</td>
<td>Frequency Dependent</td>
<td>Simulation</td>
<td>4</td>
</tr>
<tr>
<td>Bacterium</td>
<td><em>Bacillus thuringiensis</em></td>
<td>Indian meal moth <em>Plodia interpunctella</em></td>
<td>Non-linear</td>
<td>Statistical</td>
<td>5</td>
</tr>
<tr>
<td>Bacterium</td>
<td>Furunculosis (Aeromonas <em>salmonicida</em>)</td>
<td>Chinook Salmon (Oncorhynchus <em>tshawytscha</em>)</td>
<td>Density Dependent</td>
<td>Statistical</td>
<td>6</td>
</tr>
<tr>
<td>Bacterium</td>
<td><em>Pasteurir ramosa</em></td>
<td><em>Daphnia magn</em></td>
<td>Non-linear (Both Versions)</td>
<td>Statistical</td>
<td>7</td>
</tr>
<tr>
<td>Fungi</td>
<td>Anther-smut fungus (Ustilago <em>violacea</em>)</td>
<td>White campion (Silene <em>alba</em>)</td>
<td>Frequency Dependent</td>
<td>Statistical</td>
<td>8</td>
</tr>
<tr>
<td>Fungi</td>
<td><em>Batrachochytrium dendrobatidis</em></td>
<td>Mountain yellow-legged frog (<em>Rana muscosa</em>)</td>
<td>Density Dependent</td>
<td>Model fitting</td>
<td>9</td>
</tr>
<tr>
<td>Fungi</td>
<td><em>Batrachochytrium dendrobatidis</em></td>
<td>Mountain yellow-legged frog (<em>Rana muscosa</em>)</td>
<td>Density Dependent</td>
<td>Model fitting</td>
<td>9</td>
</tr>
<tr>
<td>Fungi</td>
<td>Anther-smut fungus (Ustilago <em>violacea</em>)</td>
<td>White campion (Silene <em>alba</em>)</td>
<td>Exponential frequency dependent transmission</td>
<td>Model fitting</td>
<td>10</td>
</tr>
<tr>
<td>Macroparasite (Trematode)</td>
<td><em>Schistosoma mansoni</em></td>
<td>Bioamphalaria <em>glabrata</em></td>
<td>Density Dependent (Parasite Density)</td>
<td>Statistical</td>
<td>11</td>
</tr>
<tr>
<td>Macroparasite (Trematode)</td>
<td>Diplostomum spathaceum cercariae</td>
<td>Rainbow trout <em>(Oncorhynchus mykiss)</em></td>
<td>Frequency Dependent</td>
<td>Statistical</td>
<td>12</td>
</tr>
<tr>
<td>--------------------------</td>
<td>---------------------------------</td>
<td>----------------------------------------</td>
<td>---------------------</td>
<td>------------</td>
<td>----</td>
</tr>
<tr>
<td>Macroparasite (Trematode)</td>
<td><em>Microphallus</em> sp.</td>
<td>New Zealand Mud Snail <em>Potamopyrgus antipodarum</em></td>
<td>Non-linear and linear</td>
<td>Statistical</td>
<td>13</td>
</tr>
<tr>
<td>Macroparasite (Trematode)</td>
<td><em>Centrocestus armatus</em> cercariae</td>
<td>Minnow <em>(Zacco temminckii)</em></td>
<td>Frequency Dependent</td>
<td>Statistical</td>
<td>14</td>
</tr>
<tr>
<td>Mite Nematode</td>
<td><em>Coccipolipus hippocodamiae</em></td>
<td>Two-spot ladybird <em>Adalia bipunctata</em></td>
<td>Density Dependent</td>
<td>Statistical</td>
<td>15</td>
</tr>
<tr>
<td>Nematode</td>
<td><em>Anguillicola crassus</em></td>
<td>Copepods Bank Voles <em>Clethrionomys glareolus</em></td>
<td>Non-linear</td>
<td>Statistical</td>
<td>16</td>
</tr>
<tr>
<td>Virus</td>
<td>Cowpox virus</td>
<td>Bank Voles <em>(Clethrionomys glareolus)</em> and Wood Mice <em>(Apodemus sylvaticus)</em></td>
<td>Frequency Dependent</td>
<td>Statistical</td>
<td>17</td>
</tr>
<tr>
<td>Virus</td>
<td>Cowpox virus</td>
<td>Western Tent Caterpillars <em>(Malacosoma californicum pluviale)</em></td>
<td>Frequency Dependent</td>
<td>Statistical</td>
<td>18</td>
</tr>
<tr>
<td>Virus</td>
<td>Nuclear polyhedrosis virus (PRV)</td>
<td>Domestic Pigs <em>LdNPV</em></td>
<td>Density Dependent</td>
<td>Statistical</td>
<td>19</td>
</tr>
<tr>
<td>Virus</td>
<td>Nuclear polyhedrosis virus (LdNPV)</td>
<td>Gypsy moth <em>(Lymantria dispar)</em></td>
<td>Non-linear (Power function)</td>
<td>Statistical</td>
<td>20</td>
</tr>
<tr>
<td>Virus</td>
<td>Nuclear polyhedrosis virus (LdNPV)</td>
<td>Gypsy moth <em>(Lymantria dispar)</em></td>
<td>Non-linear (Heterogeneity Function)</td>
<td>Model fitting</td>
<td>22</td>
</tr>
<tr>
<td>Virus</td>
<td>Nuclear polyhedrosis virus (LdNPV)</td>
<td>Gypsy moth <em>(Lymantria dispar)</em></td>
<td>Non-linear (Heterogeneity Function)</td>
<td>Statistical</td>
<td>22</td>
</tr>
<tr>
<td>Virus</td>
<td>Host Species</td>
<td>Description</td>
<td>Statistical Model</td>
<td>Model Fitting</td>
<td></td>
</tr>
<tr>
<td>-------</td>
<td>--------------</td>
<td>-------------</td>
<td>-------------------</td>
<td>--------------</td>
<td></td>
</tr>
<tr>
<td>Ambystoma tigrinum virus (ATV)</td>
<td>Tiger Salamanders (<em>Ambystoma tigrinum</em>)</td>
<td>Power function</td>
<td>23</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Granulosis Virus (PiGV)</td>
<td>Indian meal moth <em>Plodia interpunctella</em></td>
<td>Power Law Function and Negative Binomial</td>
<td>24</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Virus</td>
<td>Mosquito (<em>Aedes aegypti</em>)</td>
<td>Non-linear</td>
<td>25</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Invertebrate iridescent virus (IIV)</td>
<td>Rainbow trout (<em>Oncorhynchus mykiss</em>)</td>
<td>Non-linear, Saturating (Non-linear, similar to a frequency dependent power law)</td>
<td>26</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hematopoietic Necrosis Virus (IHNV)</td>
<td>Field Voles (<em>Microtus agrestis</em>)</td>
<td>Model fitting</td>
<td>27</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cowpox virus</td>
<td>Harbour Seal (<em>Phoca vitulina</em>)</td>
<td>Frequency Dependent</td>
<td>28</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Appendix 2B. Laboratory animal collection and maintenance

In both 2010 and 2011, we obtained Pacific chorus frog (*Pseudacris regilla*) eggs from field sites in California, USA. We maintained tadpoles in 40-L plastic containers with airstones and fed a 1:1 mixture of commercial fish food (TetraMin and Spirulina) with 50 percent water changes occurring every 2–3 days until tadpoles reached approximately stage 33 (Gosner 1960).

Following experimental procedures, tadpoles were maintained in 1 L containers and fed 50:50 mixture of fish food before being euthanized and preserved.

Snail first intermediate hosts (*Helisoma trivolvis*) naturally infected with *Ribeiroia ondatrae* were collected from field sites surrounding San Francisco Bay, California (Contra Costa, Alameda, Santa Clara and San Mateo counties).

Appendix 2C. Experimental procedures

For all experimental procedures, *R. ondatrae* infected snails were placed in 50-mL centrifuge tubes from 18:00 – 22:00H and to collect newly emerged cercariae. All experiments were started at 24:00H when cercariae were less than 6 hours old to minimize any differences due to cercariae age and infectivity (Karvonen et al. 2003, Paller et al. 2007). Cercariae were counted using a glass pipette under a dissecting microscope into 2-mL vials before being added to experimental containers filled with treated tap water. Exposures took place in a temperature control room at 22°C. Tadpoles were acclimated to the experimental containers for 30 minutes to allow them to resume normal activity prior to adding *R. ondatrae* cercariae. Each treatment was replicated 10 times. Experimental containers were selected to maintain the same depth (6 mm), but varied in length and width in the experiment where volumes were manipulated (range 6 X 6 mm to 42 X 29 mm).
We used ANOVA with Tukey HSD for all pairwise comparisons of tadpole wet mass (mg) and developmental stage (Gosner 1960, Table 2.6) across experiments. Tadpoles used in the experiment examining host density were significantly larger (p < 0.0264) than those used in all other experiments, which did not differ significantly from each other (p > 0.247). We found that tadpoles used in manipulations of time, parasite density and parasite number were significantly further in development than the tadpoles used in the experiment varying host density (p < 0.001). Likewise tadpoles in parasite density and number experiments were significantly further in development than those used in the manipulation of host behavior (p < 0.0175). However, the mean developmental stage across each experiment only varied from 35.2 to 35.9, less than one stage, so we believe that stage was maintained consistently across experiments.
Table 2.6. Summary of wet mass (± standard error) and developmental stages\(^a\) (± standard error) of Pacific Chorus frog tadpoles (*Pseudacris regilla*) used in experimental procedures to assess transmission dynamics of the trematode *Ribeiroia ondatrae*.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Wet mass (mg) ± SE</th>
<th>Developmental Stage ± SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time</td>
<td>702.0 ± 55.7</td>
<td>35.8 ± 0.4</td>
</tr>
<tr>
<td>Parasite Number</td>
<td>668.0 ± 67.2</td>
<td>35.8 ± 0.4</td>
</tr>
<tr>
<td>Parasite Density</td>
<td>661.2 ± 58.9</td>
<td>35.9 ± 0.4</td>
</tr>
<tr>
<td>Host Density</td>
<td>769.8 ± 86.5</td>
<td>35.2 ± 0.2</td>
</tr>
<tr>
<td>Host Behavior</td>
<td>640.2 ± 79.7</td>
<td>35.4 ± 0.4</td>
</tr>
</tbody>
</table>

\(^a\)(Gosner 1960)
Appendix 2D. Amphibian anesthesia

We used a dilute solution of MS-222 to reduce the role of tadpole anti-parasite behavior, thus isolating the role of parasite behavior in transmission dynamics. Our methods followed Daly and Johnson (2010) and were effective in removing anti-parasite behaviors of the tadpoles for approximately 25–30 minutes during exposure. The numbers of replicates for the different volume treatments varied from 4–7 depending on the numbers of tadpoles that remained anesthetized for the required 25–30 minutes or did not recover from anesthesia.

Appendix 2E. Amphibian necropsy

Tadpoles were euthanized with buffered MS-222 (Tricaine methanesulfonate, Western Chemical Inc.) and preserved in 10% buffered formalin until necropsy, where we inspected all external surfaces and removed and examined all muscle tissue and organs using a dissecting microscope. Metacercariae were examined under a compound microscope to observe distinguishing features to allow for species identification (Schell et al. 1985, Johnson and McKenzie 2009, Szuroczki and Richardson 2009).

Appendix 2F. Previous empirical data

We collected data representing the number of parasites infected or attached to hosts based on experiments varying parasite density and parasite number using Plot Digitizer 2.5 (Karvonen et al. 2003 Figure 4A&B, Paller et al. 2007 Figure 3A&B). If a point was not distinguishable from other points because they overlapped those points were not included in the analyses.
CHAPTER 3

PARASITE TRANSMISSION IN COMPLEX COMMUNITIES: PREDATORS AND ALTERNATIVE HOSTS ALTER PATHOGENIC INFECTIONS IN AMPHIBIANS


Abstract

While often studied in isolation, host-parasite interactions are typically embedded within complex communities. Other community members, including predators and alternative hosts, can therefore alter parasite transmission (e.g., the dilution effect), yet few studies have experimentally evaluated more than one such mechanism. Here, we used data from natural wetlands to design experiments investigating how alternative hosts and predators of parasites mediate trematode (*Ribeiroia ondatrae*) infection in a focal amphibian host (*Pseudacris regilla*). In short-term predation bioassays involving molluscs, zooplankton, fish, larval insects, or newts, four of seven tested species removed 62 to 93% of infectious stages. In transmission experiments, damselfly nymphs (predators) and newt larvae (alternative hosts) reduced infection in *P. regilla* tadpoles by ~50%, whereas mosquitofish (potential predators and alternative hosts) did not significantly influence transmission. Additional bioassays indicated that predators consumed parasites even in the presence of alternative prey. In natural wetlands, newts had similar infection intensities as *P. regilla*, suggesting they commonly function as alternative hosts despite their unpalatability to downstream hosts, whereas mosquitofish had substantially lower infection intensities and are unlikely to function as hosts. These results underscore the importance of studying host-parasite interactions in complex communities and of broadly linking research on predation, biodiversity loss, and infectious diseases.
Introduction

While often studied in isolation, parasites and hosts interact with members of a diverse community that can influence patterns of infection and pathology (Hopper et al. 2008). The hypothesis that community diversity, including host and non-host species, leads to reduced pathogen transmission has been termed the ‘dilution effect’ (Keesing et al. 2006, 2010). Previous research on the dilution effect has focused primarily on vector-borne microparasites (e.g., West Nile virus and the Lyme-causing bacterium, *Borrelia burgdorferi* (Ostfeld and Keesing 2000a,b, LoGiudice et al. 2003, Ezenwa et al. 2006)). In these cases, additional vertebrate host diversity is hypothesized to increase the number of instances in which infected vectors bite low-competency species, thereby lowering the number of successful transmission events (“encounter reduction” Keesing et al. 2006).

However, the occurrence of alternative, low-competency hosts may be but one of several pathways through which community members influence parasite transmission to suitable hosts. Because most studies of the dilution effect have been correlational, typically comparing richness to a measure of infection risk, it is often not clear what ecological mechanisms are responsible (Allan et al. 2003, Ezenwa et al. 2006). In particular, predators in a community can alter infections either by consuming hosts (susceptible host regulation) or by preying upon parasite infectious stages (encounter reduction) (Ostfeld and Holt 2004, Johnson et al. 2010). Given the high frequency of predator-parasite links in aquatic food webs, predation upon parasites is likely a common occurrence with the potential to influence transmission dynamics (Lafferty et al. 2008, Johnson et al. 2010). This is especially likely to occur for parasites that depend on free-living stages to move between hosts, which include many trematodes, cestodes, and nematodes (Johnson and Thieltges 2010). Indeed predation of parasites has been used as a
method of biological control for schistosomiasis in humans (Siau et al. 1992) and helminths of livestock (Nichols et al. 2008). In some cases, multiple mechanisms could operate simultaneously, e.g., alternative hosts ‘distract’ parasites away from focal hosts while predators reduce the abundance of free-living stages. These observations underscore the importance of evaluating the dilution potential of multiple species in nature and assessing how they affect transmission to sensitive hosts.

To evaluate the influence of ecological communities on parasite transmission and infection risk, we focused on interactions between the pathogenic trematode *Ribeiroia ondatrae*, a sensitive amphibian host (*Pseudacris regilla*), and other vertebrate and invertebrate members of the community. Our primary objectives were to: 1) quantify the ability of diverse aquatic species (crustaceans, molluscs, insects, fish and amphibians) to reduce the abundance of *R. ondatrae* free-living stages ( cercariae) and 2) examine the effect of a subset of species on parasite transmission to focal host tadpoles by serving as predators or alternative hosts. To increase the biological relevance of our study, we utilized existing field data to identify aquatic free-living species to use in laboratory trials and compared infection patterns of two vertebrate taxa between experimental and field data. Our study aimed to identify the relative importance of multiple underlying biological mechanisms (i.e., consumption, infection, or both) that can lead to changes in disease risk. Improving our understanding of the individual mechanisms through which species diversity influences disease dynamics has important consequences for human and wildlife health in a period of biodiversity loss.

**Methods**

*Study system*
The trematode *Ribeiroia ondatrae* has a complex life cycle involving sequential infection of planorbid snails, larval amphibians or fish, and aquatic birds (Johnson et al. 2004). Free-living cercariae released by infected snails typically locate an amphibian larva, penetrate the developing limb buds, and develop into metacercariae, which can cause limb malformations or mortality (Johnson et al. 1999). The parasite is trophically transmitted to definitive host birds through consumption of infected amphibians. In the western United States, Pacific chorus frogs (*Pseudacris regilla*) are a common host for *R. ondatrae* that frequently exhibit severe pathology (Johnson et al. 1999, 2002), making them an appropriate focal host.

**Predation bioassays**

We examined the abilities of seven aquatic species to remove *R. ondatrae* cercariae in laboratory trials. These included two vertebrates [*California newts* (*Taricha torosa*) and western mosquitofish (*Gambusia affinis*)] and five invertebrates [*California clam shrimp* (*Cyzicus californicus*), backswimmers (*Notonecta* sp.), damselfly nymphs (*Lestes* sp. and *Enallagma* sp.), dragonfly nymphs (*Anax* sp.), and clams (*Sphaerium* sp.)]. Selection of these taxa was based on their co-occurrence with *R. ondatrae* in natural wetlands, their suspected potential to remove parasites via multiple mechanisms (i.e., consumption and successful transmission), and prior knowledge of the ability of related taxa to consume cercariae in other systems (Schotthoefer et al. 2007, Thieltges et al. 2008, Kaplan et al. 2009). In selecting these species, we utilized existing field survey data on the occurrence of mosquitofish, amphibians and invertebrates from 49 ponds that contained *R. ondatrae* in the San Francisco Bay Area of California (see also Johnson and Buller 2011). Field methods and the frequency of co-occurrence of each of the seven taxa in ponds supporting *R. ondatrae* are provided in Appendix 3A.
We developed laboratory bioassays to determine the degree to which each of the selected species reduced the abundance of free-living cercariae and the mechanism(s) through which such effects occurred. The body sizes and sample sizes of species used in bioassays are provided in Table 3.1 and methods for animal collection and maintenance in Appendix 3B. Each individual was fasted for 24 hr prior to assay. We isolated cercariae from field-collected snails by placing them individually in 50-mL centrifuge tubes over night. For each assay, we placed a single individual of each species in 60 ml of water with 30 *R. ondatrae* cercariae for 30 min, after which we removed the individual and counted any remaining cercariae. To assess parasite recovery in the absence of predators or hosts, we included containers with parasites and no other species. Forty-eight hours following trial completion, we necropsied any vertebrate hosts (mosquitofish, chorus frog tadpoles, and newts) to quantify the number of encysted parasites, thereby allowing us to evaluate the roles of infection and consumption as two distinct mechanisms. We also dissected a subset of damselflies (*n* = 3), clam shrimp (*n* = 5) and clams (*n* = 5) 2 days after completing the trials to confirm that metacercariae did not infect invertebrates (Schotthoefer et al. 2007). Lastly, we explored the propensity of two predators to eat cercariae in the presence of alternative prey. Damselfly nymphs (*Enallagma* spp.) and mosquitofish were offered cercariae and *Daphnia middendorffiana* simultaneously at three prey densities. We used a prey selectivity index to quantify preference (Chesson 1983; see Appendix 3C).

**Transmission experiments**

Building from the laboratory bioassays, we selected three of the species most effective at removing *R. ondatrae* cercariae to evaluate their effects on parasite transmission to chorus frog tadpoles. We exposed individual tadpoles to 30 cercariae either alone (control) or in the presence
Table 3.1. The hypothesized interaction between aquatic taxa and the frequency that each taxa occurred in ponds that support *R. ondatrae* in the Bay Area of California (*n* = 49 ponds) including the number of individuals of each species examined for the ability to reduce the number of *Ribeiroia ondatrae* cercariae in laboratory trials (N) and the range of body size of the specimens.

<table>
<thead>
<tr>
<th>Taxa</th>
<th>Potential Interaction with <em>R. ondatrae</em></th>
<th>Frequency of Co-occurrence with <em>R. ondatrae</em> (%)</th>
<th>n</th>
<th>Body Length ± SE (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Invertebrate</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Backswimmers <em>Notonecta</em> sp.</td>
<td>Predator</td>
<td>84</td>
<td>20</td>
<td>8.50 ± 0.32</td>
</tr>
<tr>
<td>California Clam Shrimp <em>Cyzicus californicus</em></td>
<td>Predator</td>
<td>6</td>
<td>11</td>
<td>14.34 ± 0.10</td>
</tr>
<tr>
<td>Clams <em>Sphaerium</em> sp.</td>
<td>Predator</td>
<td>4</td>
<td>10</td>
<td>8.429 ± 0.05</td>
</tr>
<tr>
<td>Damselfly nymphs <em>Lestes</em> sp. and <em>Enallagma</em> sp.</td>
<td>Predator</td>
<td>90</td>
<td>8</td>
<td>17.93 ± 0.94</td>
</tr>
<tr>
<td>Dragonfly nymphs <em>Anax</em> sp.</td>
<td>Predator</td>
<td>80</td>
<td>14</td>
<td>34.69 ± 1.44</td>
</tr>
<tr>
<td><strong>Vertebrate</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pacific Chorus Frog <em>Psuedacris regilla</em></td>
<td>Target Host</td>
<td>n/a</td>
<td>16</td>
<td>14.27 ± 1.14</td>
</tr>
<tr>
<td>California Newt <em>Taricha torosa</em></td>
<td>Predator/Alternative Host</td>
<td>78</td>
<td>18</td>
<td>17.45 ± 0.43</td>
</tr>
<tr>
<td>Western Mosquitofish <em>Gambusia affinis</em></td>
<td>Predator/Alternative Host</td>
<td>10</td>
<td>20</td>
<td>20.82 ± 0.09</td>
</tr>
</tbody>
</table>
of an individual damselfly (*Enallagma* spp. or *Lestes* sp.), mosquitofish, or newt in 1 L of water. Trials lasted for 1 hr with 16 replicates of each treatment (sizes of all species provided in Appendix 3D). Because these species could be amphibian predators and thus alter the behavior of tadpoles with potential effects on infection (Thiemann and Wassersug 2000, Gunzberger and Travis 2005), we also included effluent treatments in which we added cercariae and 50 ml of water from containers housing damselflies (*n* = 8 replicates), mosquitofish (*n* = 8 replicates) or newt (*n* = 16 replicates). Forty-eight hours after the end of each trial, we quantified metacercariae within chorus frog tadpoles, newts, and mosquitofish to evaluate the relative contributions of predation-mediated, behavior-mediated, and infection-mediated changes in transmission to chorus frogs (see Appendix 3E for necropsy procedures). To examine whether infection patterns from laboratory transmission trials were consistent with field infection patterns, we collected newts (*n* = 46), mosquitofish (*n* = 25), and chorus frogs (*n* = 170) from 10 sites in California. Specimens were necropsied and examined for *R. ondatrae* metacercariae as above.

*Statistical analysis*

We used generalized linear mixed models (Zuur et al. 2009) to test the effects of each species in the bioassays on the fate of each administered parasite (removed [eaten or transmitted] vs. remaining at the end of 30 min). Specifically, we used the R package lme4 and the Laplace approximation method (R Development Core Team 2008) with a binomially distributed error and logit-link function to evaluate the influence of species (main effect) with individual predator identity as a random effect to account for variation beyond what we could control (i.e., the fate of parasites within the same trial may not be independent due to predator satiation, individual
differences among predators, etc.). To further differentiate the relative roles of infection and consumption for vertebrates in the bioassay, we analyzed the fate of removed cercariae (encysted or not) with species as a main effect and individual predator identity as a random effect. To evaluate the roles of predators and alternate hosts on *R. ondatrae* transmission to chorus frogs and total infection, we used ANCOVA with tadpole stage as a covariate and damselflies, mosquitofish or newts as fixed effects. For field and laboratory data, we used paired t-tests to compare infections between co-occurring species (e.g., newts or mosquitofish, and chorus frogs). Additionally, we conducted an unpaired t-test on the ratios of infection in newts and chorus frogs to assess whether laboratory infections differed from those in the field.

**Results**

*Predation experiments*

We recovered all cercariae in control treatments without predator or alternative host species present (Figure 3.1A), illustrating the effectiveness of our method in tracking parasites. Among bioassayed species, parasite removal varied significantly, ranging from 0% to 93% (Figure 3.1A). Four of the seven tested species removed > 60% of parasites, with the greatest removal by mosquitofish (*Z* = 12.221, *p* < 0.0001), followed by newt larvae (*Z* = 10.435, *p* < 0.0001), damselflies (*Z* = 7.458, *p* < 0.0001), and clam shrimp (*Z* = 7.573, *p* < 0.0001, Figure 3.1A). Dragonfly nymphs, backswimmers, and clams had no significant effect on cercarial abundance (*Z* < 1.116, *p* > 0.264, Figure 3.1A). We also observed differences in whether effects stemmed from consumption, infection, or both. While damselflies, clam shrimp, and clams showed no evidence of infection at the end of trials, ~57% of administered cercariae were recovered as metacercariae from both chorus frogs and newts with no significant difference between the two
species (Z = -0.174, p = 0.862, Figure 3.1B). Interestingly, however, the total recovery of parasites (sum of leftover cercariae and encysted metacercariae) was significantly lower for newts than chorus frogs (Z = -2.817, p = 0.005, Figure 3.1B). Although, we cannot differentiate failed infection from consumption, metacercariae were frequently recovered from the oral cavity of newts, suggesting that consumption may have been responsible for the difference. For mosquitofish, <5% of removed cercariae were recovered as metacercariae, suggesting that the primary mechanism was consumption (Z = -9.007, p < 0.0001, Figure 3.1B). This notion is further supported by direct observations of mosquitofish consuming cercariae and the low number of cercariae tails recovered at the end of the trials (1.3 ± 0.1 SE), which are indicative of attempted infection. Finally, damselflies and mosquitofish in the alternative prey trials still consumed cercariae in the presence of Daphnia; mosquitofish consumed all available prey (cercariae and Daphnia), while damselflies ate ~33% of each prey type in proportions similar to their availability (see Appendix 3C for detailed results).

Transmission experiments

Damselflies reduced transmission of R. ondatrae to tadpoles by ~50% compared to trials with tadpoles alone (F_{4,59} = 2.67, p = 0.041; Tukey post-hoc, p = 0.015, Figure 3.2A). There was no difference in tadpole infection between treatments with damselflies and those with damselfly effluent (Tukey post-hoc, p = 0.735). Mosquitofish did not influence transmission relative to controls (Tukey post-hoc, p = 0.564) or their effluent (Tukey post-hoc, p = 1.000). We recovered an average of 0.4 ± 0.2 (SE) metacercariae per fish and 9.2 ± 1.4 (SE) in tadpoles, indicating a strong preference of R. ondatrae to infect tadpoles in the presence of both species. Newts also
Figure 3.1. A. Average percent of 30 *Ribeiroia ondatrae* cercariae removed by a single alternative host or predator. B. Average number of *R. ondatrae* cercariae that were recovered alive after a 30-minute trial (lower case letters), the average number of cercariae recovered as metacercariae (upper case letters) from each species, and the number eaten or died. Letters represent statistically significant differences. Error bars ± 1 standard error.
Figure 3.2. A. Average number of *Ribeiroia ondatrae* metacercariae recovered from Pacific chorus frog (*Pseudacris regilla*) tadpoles following exposure to cercariae alone or in the presence of an individual damselfly nymph or mosquitofish (*Gambusia affinis*), or 50mL of damselfly effluent or mosquitofish effluent. B. Metacercariae recovered from tadpoles following exposure in the presence of an individual California newt (*Taricha torosa*) larvae, or 50mL of newt effluent, or control with newt absent. Note differences between y-axes. Letters represent statistically significant differences. C. Average number of *R. ondatrae* metacercariae recovered from mosquitofish (circles) and California newt larvae (squares) plotted against the number of metacercariae in co-occurring Pacific Chorus frog tadpoles collected from 10 sites in the San Francisco Bay Area of California, USA in 2010. Dashed line represents the 1:1 relationship of equal infection intensity in both species. Error bars ± 1 standard error.
reduced transmission by ~50% relative to tadpoles alone or with newt effluent (F$_{3,47}$ = 25.99, p < 0.0001; tadpole stage[covariate] p = 0.0003; Tukey post-hoc, p < 0.0001, Figure 3.2B).

However, newts exhibited comparable infections relative to cohabitating tadpoles (p = 0.094), and the sum of metacercariae in both newts and tadpoles did not differ significantly from control tadpoles (p = 0.851). Trials with damselflies and fish were analyzed separately from trials with newts because there was a significant difference between infection intensity in the controls between the two experiments (t$_{31}$ = 4.43, p = 0.0001 Figure 3.2A and B), likely arising from variation between years.

Average *R. ondatrae* infection in mosquitofish collected from natural wetlands ranged from 0.2 – 1.2 metacercariae per fish compared with 2.3 – 76.6 metacercariae per chorus frog from the same wetland, representing a 193% average difference between the species (paired t-test, t$_{3}$ = 3.88, p = 0.030, Figure 3.2C). However, newt infection intensity did not differ from co-occurring chorus frogs (t$_{8}$ = -0.86, p = 0.417, Figure 3.2C), with an overall average of 23.0 ± 2.4 (SE) metacercariae per newt and 17.7 ± 5.2 (SE) per tadpole across sites. In addition, the ratios of infection between the laboratory trials and the field data were comparable (t$_{24}$ = 0.80, p = 0.429).

**Discussion**

Our results indicate that diverse members of ecological communities consume or interfere with the movement of pathogenic trematode cercariae to sensitive amphibian hosts. By carefully tracking the fate of each administered parasite in laboratory trials, we were able to experimentally assess multiple mechanisms of the dilution effect, including predation on infectious stages, the role of alternative hosts, and the combination of the two. Through
laboratory bioassays informed by field data, we identified a range of vertebrate and invertebrate predators with varying abilities to remove cercariae. Several feeding modes were effective for consuming cercariae, including active (mosquitofish), ambush (damselfly larvae), and filter feeding (clam shrimp) (see also Schotthoefer et al. 2007, Thieltges et al. 2008, Kaplan et al. 2009). Subsequent bioassays with mosquitofish and damselflies showed that both predators consumed cercariae when offered *Daphnia middendorffiana* as alternative prey, suggesting that cercariae are likely to be consumed even in complex communities with multiple prey species. Because we tracked the fate of each parasite to determine whether it was removed (consumed or died), became encysted, or remained free-swimming at the end of trials, our bioassays allowed clear differentiation among potential dilution mechanisms.

Building on bioassay results, our transmission experiments showed that predators and alternative hosts reduced *R. ondatrae* transmission to sensitive tadpole hosts by as much as 50%. The presence of newts did not reduce the cumulative recovery of administered parasites (summed among hosts), but altered their fate, diverting infection from chorus frogs to newts, which are less-suitable hosts (see below). Damselflies consumed a substantial fraction of administered parasites and thus prevented them from infecting tadpole hosts. Because chorus frogs are highly sensitive to *R. ondatrae* infection, often exhibiting mortality or developmental malformations (Johnson et al. 1999, 2002, Goodman and Johnson 2011), such changes in transmission could have important effects on host fitness in nature. Surprisingly, mosquitofish – which had strong effects in the bioassay trials – did not significantly alter transmission when paired with a tadpole. This result may stem from methodological differences between the bioassay and transmission trials; the transmission trials used a large container volume but the same number of cercariae, such that the lower cercarial density could explain observed patterns
if fish predation is density dependent (Murdoch et al. 1975). Alternatively or additionally, parasites could have infected the tadpole before fish were able to consume them, effectively nullifying the role of predation. Predator effluent did not alter parasite transmission, although predators were not fed tadpoles prior to effluent collection, which can influence behavioral responses (Relyea and Werner 1999).

Our laboratory results have important implications for natural communities. Data from natural wetlands indicate that many of the examined species commonly co-occur with *R. ondatrae* and thus have the potential to influence transmission. In particular, members in this community may influence disease because *R. ondatrae* is known to cause mortality and malformations in a variety of amphibian species (Johnson et al. 2002). Our field data from California wetlands indicated that newts commonly co-occur with chorus frogs and often support similar infection levels. Interestingly, however, *R. ondatrae* infecting newts might often be unsuccessful at reaching a definitive host because of the potent tetrodotoxin that prevents birds from preying on adult newts, thus inhibiting parasite transmission after the newt's larval stage (Brodie 1968, Mobley and Stidham 2000, Gunzberger and Travis 2005). Despite this, cercariae in laboratory trials showed no preference for chorus frog tadpoles over newt larvae. This is in contrast with the pattern of mosquitofish, which despite co-occurring with chorus frogs and *R. ondatrae*, rarely became infected in the laboratory or the field, indicating a strong preference of the parasite for amphibians. Although fish can also mount an immune response against invading *Ribeiroia* cercariae (Huizinga and Nadakavukaren 1997), our laboratory experiments indicated clearly that parasites were preferentially infecting tadpoles rather than encysting on fish and being subsequently eliminated by immune activation. More research is needed to assess the relative importance of diversity, and of different dilution mechanisms, on transmission in natural
The mechanism by which predators reduce parasite transmission helps to link the dilution effect and a growing interest in predation and disease dynamics. Predators have been considered important mediators of disease through their effects on susceptible host regulation or preferential removal of infected hosts (Packer et al. 2003, Ostfeld and Holt 2004, Johnson et al. 2006), or consumption of insect vectors (Moore et al. 2009). Our study further indicates that predation can alter transmission through removal of infective stages, consistent with previous studies that have identified predation as an important mechanism of the dilution effect (Hopper et al. 2008, Thielges et al. 2008, Prinz 2009, Johnson et al. 2010). Predator-parasite links in food webs, which are common in many systems, can have important consequences for predator diets and resource flow (Kuris et al. 2008, Lafferty et al. 2008), such that quantifying the consumption of parasites in nature presents a promising future research direction (Kaplan et al. 2009). Taken together, these results demonstrate that, alongside the host community, predator community composition can strongly influence patterns of infection. Given widespread increases in diseases of humans and wildlife concurrent with rapid losses in biodiversity (Daszak et al. 2000) investigating changes in community composition has important consequences for understanding pathogenic infections in diverse taxa (Keesing et al. 2010).

Appendices

Appendix 3A. Field survey methods

Survey sites were located in four counties surrounding San Francisco Bay, California (Contra Costa, Alameda, Santa Clara and San Mateo counties). Habitat surrounding the ponds consisted of oak woodland and chaparral, while land use in the area included grazing, recreational areas,
and natural preserves. Each pond \((n = 49)\) was visited once in May/June and again in July/August of 2009 and 2010. Detection of fish, amphibians and invertebrates was accomplished using a combination of visual encounter surveys around the pond margin, net sweeps, and seines in deeper pond regions (Heyer et al. 1994, Dodd 2010). We conducted net sweeps perpendicular to shore every three to five meters around the margin of the pond using a D-net (1.4 mm mesh, 2600 cm\(^2\) opening). We used a seine net (4 mm mesh, 1 m high by 2 m wide) to conduct three or four collections of approximately five meters in length in each pond. We identified and counted all species of captured amphibians, fish and invertebrates during net sweeps and seines before releasing them back into the pond. To determine which sites supported \textit{Ribeiroia ondatrae} infection, we collected 10 recently metamorphosed \textit{Pseudacris regilla} tadpoles from each pond to be necropsied in the laboratory, where parasite prevalence and numbers of metacercariae were quantified. Previous work has shown this sample size to be adequate to detect \textit{R. ondatrae} at the pond scale (Hartson et al. 2011). The frequency of co-occurrence of each of the seven taxa in ponds supporting \textit{R. ondatrae} are provided in Table 3.1.

To examine whether the patterns of infection observed in the laboratory trials were consistent in nature, we collected mosquitofish from 4 sites \((n = 25, \text{ body length } 30.8 \text{ mm } \pm 0.8 \text{ SE})\) and \textit{T. torosa} from 7 sites \((2 \text{ sites sampled in both } 2009 \text{ and } 2010, n = 46, \text{ snout-vent length } 18.8 \text{ mm } \pm 0.5 \text{ SE})\). We collected tadpoles \((n = 79, \text{ snout-vent length } 14.0 \text{ mm } \pm 0.4 \text{ SE}, \text{ Gosner stages } 28-45)\) or recently emerged metamorphs \((n = 91, \text{ snout-vent length } 17.3 \text{ mm } \pm 0.3 \text{ SE}, \text{ Gosner stage } 46)\) of \textit{P. regilla} from these sites.
Appendix 3B. Laboratory Animal collection and maintenance

We collected alternative hosts and predators in June–August 2009 and 2010 in central California during field surveys, with the exception of backswimmers, which we collected from cattle tank mesocosms in Boulder, Colorado in June 2010. We shipped California specimens to the laboratory at University of Colorado Boulder where we conducted the experiments. We maintained invertebrates in 1-L containers with dechlorinated tap water (AmQuel plus and NovAqua plus, Kordon®) and fed algae (clam shrimp) or Daphnia (damselflies, dragonflies, and backswimmers). We fed newt larvae chopped California blackworms (Lumbriculus variegatus) and mosquitofish commercial fish food (TetraMin, Tetra®). The number of individuals of each species examined laboratory trials (N) and the range of body size of the specimens are presented in Table 3.1.

In both 2009 and 2010, we obtained chorus frog eggs from field sites in California. We maintained tadpoles in 40-L plastic containers with airstones and fed a 1:1 mixture of commercial fish food (TetraMin and Spirulina) with 50 percent water changes occurring every 2–3 days until tadpoles reached approximately stage 33 (Gosner 1960).

Appendix 3C. Alternative prey bioassay methods and results.

Methods

We conducted additional bioassay trials to assess the ability of mosquitofish (Gambusia affinis) or damselfly nymphs (Enallagma spp.) to consume Ribeiroia ondatrae cercariae in the presence of alternative prey. Each predator was fasted for 24 hr. prior to assay to standardize hunger and all trials were conducted in the morning (0200-0600hr) corresponding to peak cercarial shedding.

We had three treatments: 20 R. ondatrae cercariae and 10 Daphnia middendorffiana, 15 of each
taxa, and 10 *R. ondratae* cercariae with 20 *D. middendorfiana*. Each treatment was replicated five times for each species. Each individual was placed into 60ml of water for 30 minutes of acclimation before the prey were added. After 30 minutes, predators were removed and the remaining prey of each taxa were immediately counted using a dissecting microscope. We included replicates of each treatment with no predator present to ensure we could recover all prey items offered as a control treatment to assess parasite recovery.

To determine whether predators showed a prey preference for *Daphnia* or cercariae, we calculated the ‘case 2’ form of Chesson’s α, which is a prey selectivity index that is appropriate for situations when prey is removed without replacement (Chesson 1978, Chesson 1983).

Chesson’s α was calculated using the index:

\[
\alpha_i = \frac{\ln((n_{i0} - r_i)/n_{i0})}{\sum_{j=1}^{m} \ln((n_{j0} - r_j)/n_{j0})}, \quad i = 1, ..., m
\]

where \( n_{i0} \) is the number of individuals of prey type \( i \) present at the beginning of the trial, \( r_i \) is the number of individuals of prey type \( i \) remaining at the end of the trial and \( m \) is the number of prey types. Values for \( \alpha_i \) can range between zero (perfect prey avoidance) and one (perfect prey selection). We used a one-sample t-test to compare the mean Chesson's α value for cercariae to the expected value of \( 1/m \) (i.e, 0.5 with two prey types), which is the value of Chesson's α that is expected with random prey selection.

**Results**

Fewer than 27% of the *Daphnia* and 33% of the cercariae were consumed in any one of the damselfly predation trials. Chesson's α for cercariae consumed by damselflies was 0.57 (SE = 0.09), which was not significantly different from the null value of 0.5 under random prey selection (\( p = 0.44, t = 0.79, df = 16 \)). This result suggests that damselflies are likely to prey on cercariae when alternative prey such as *Daphnia* are available. Mosquitofish in the alternative
prey trials with *Daphnia* and cercariae consumed all of the available prey in 14 out of the 17 successful trials. Of the trials where prey items were not completely depleted, there was one trial with a single remaining *Daphnia*, one trial with two remaining cercariae, and one trial with one *Daphnia* and five remaining cercariae. Because the prey were depleted in most of the trials, we did not use Chesson's $\alpha$ to calculate prey selectivity for the mosquitofish. While these results do not allow us to determine whether cercariae or *Daphnia* are preferred prey, it nonetheless indicates that cercariae are still consumed when other prey are available simultaneously and the mosquitofish have not been fasted prior to being offered cercariae.

*Appendix 3D. Sizes of species used in transmission laboratory experiment.*

For the laboratory transmission experiment, tadpoles ranged in stage from 33–38 (Gosner 1960), had an average mass of $651.3 \pm 17.2$ mg (SE), and did not differ significantly among treatments (stage: $p > 0.460$, mass: $p > 0.127$). The mosquitofish used in the experiment had an average body length of $20.6 \pm 0.7$ mm and a mass of $206.6 \pm 17.8$ mg (SE). The damselfly nymphs had an average body length of $14.6 \pm 1.0$ mm (SE).

*Appendix 3E. Methods for vertebrate necropsy.*

Vertebrates were euthanized with buffered MS-222 (Tricaine methanesulfonate, Western Chemical Inc.) and preserved in 10% buffered formalin until necropsy, where we inspected all external surfaces and removed and examined all muscle tissue and organs using a dissecting microscope. Metacercariae were examined under a compound microscope to observe distinguishing features to allow for species identification (Johnson and McKenzie 2009, Szuroczki and Richardson 2009).
Abstract
Understanding the effects of predation on disease dynamics is increasingly important in light of the role ecological communities can play in host-parasite interactions. Surprisingly, however, few studies have characterized the direct interactions between parasites and predators. Here we used a novel experimental approach to show that consumption of free-living parasite stages is highly context dependent, with significant influences of parasite size, predator size and foraging mode, as well as environmental condition. Larger parasites were most vulnerable to predation and small-bodied predators consumed the most infectious stages during experimental trials; however, these results depended strongly on light availability and predation strategy, emphasizing the importance of parasite traits in affecting predator detection. Intriguingly, for predators that could also become infected directly, predation functioned to help limit a predator’s infection intensity. Using established theory for predation on zooplankton, we provide a predictive framework for when predators should significantly influence parasite transmission, including the roles of body size, visibility, and predation modes. Our work contributes to understanding transmission in natural systems, the role of predator-parasite links in food webs, and the evolution of parasite morphology and behavior.
Introduction

Broader recognition of the community context of host-pathogen interactions is critical for understanding disease dynamics in natural systems. Predation can be an important factor influencing disease dynamics across diverse host-pathogen systems. Many of the effects of predation are indirect, in which predation influences host population densities leading to changes in transmission, or when selective predators remove infected individuals from the population, thereby reducing infection risk for other hosts (Packer et al. 2003, Ostfeld and Holt 2004, Borer et al. 2009). For instance, loss of vertebrate predators can increase the number of infected rodent prey (Ostfeld and Holt 2004). Predation can also induce changes in host traits such as behavior, susceptibility, or morphology leading indirectly to changes in transmission or pathology (Johnson et al. 2006, Keesing et al. 2006, Belden and Wojdak 2011, Duffy et al. 2011).

However, predators can also affect pathogen transmission directly. On one hand, predators can become infected by parasites acquired during foraging (i.e., trophic transmission; Lafferty 1999, Hall et al. 2007). On the other, predators can directly consume parasites and thereby reduce transmission or persistence. Examples range from grooming of ectoparasites to consumption of free-living infectious stages encountered in the environment (Hopper et al. 2008, Thieftges et al. 2008, Prinz et al. 2009, Johnson et al. 2010, Orlofske et al. 2012). For example, predation of trematode parasites by aquatic invertebrate reduced transmission to tadpole hosts by 50% (Orlofske et al. 2012). Thus far, however, there has been limited attention on the role of direct predation on parasites and its importance for both transmission and predator diets.

Consumer-resource theory may help provide a framework for predicting when predation is important to parasite transmission dynamics (Johnson et al. 2010). For example, there are striking differences in the ways that aquatic predators respond to zooplankton prey on the basis
of their foraging modes and body size, prey body size, and environmental conditions that influence prey detection (Brooks and Dodson 1965, Zaret 1980). Vertebrate predators, such as fish, are generally active foragers that rely on visual cues for prey detection, often leading to a positive relationship between prey size and consumption, particularly when visibility is high (Zaret 1980, Hanazato and Yasuno 1989). Alternatively, invertebrate predators, including many aquatic insects, are size-dependent predators that exhibit a non-linear relationship of consumption with prey size, such that small prey is avoided and large prey is too difficult to capture (Dodson 1974, Zaret 1980, Hanazato and Yasuno 1989). Because they are tactile, ambush foragers, the amount of light in the environment may have less of an influence on prey detection (Peckarsky 1982). However, whether these same traits and environmental factors affect the predation of parasites in aquatic ecosystems remains unexplored (Morely 2012).

Here, we used an experimental approach to examine the effects of predator and parasite species traits on consumption of parasites under different environmental conditions. We tested the capacity of two dominant aquatic predator groups, fish and insects, to consume free-living infective stages of trematode parasites. Building from consumer-resource theory, we investigated the following questions: 1) How do vertebrate and invertebrate predators with differing foraging modes respond to different parasite species as prey? 2) What is the role of parasite (prey) and predator body size in consumer interactions? 3) How are these effects moderated by environmental conditions that influence prey visibility? Our study aimed to provide a more predictive framework for understanding how predation can lead to changes in disease risk, using the relative importance of species traits on consumer–resource interactions.
Methods

Study System

We used four species of trematodes for our experimental procedures: *Cephalogonimus americanus*, *Echinostoma trivolvis*, *Ribeiroia ondatrae*, and a distinct morphotype “Magnacauda”. These species vary broadly in size and in whether they depend on direct penetration (*C. americanus*, *E. trivolvis*, *R. ondatrae*) or ingestion (Magnacauda) for transmission (Appendix 4A; Table 4.1). All species have complex life cycles involving sequential infection of planorbid snails as first intermediate hosts and larval amphibians as second intermediate hosts. However, Magnacauda and *Echinostoma trivolvis* can also use fish and snails as second intermediate hosts, respectively. Transmission occurs through free-living infective stages (cercariae) that infect second intermediate hosts and await trophic transmission to bird or mammal definitive hosts.

We examined predation by two types of aquatic organisms, fish and invertebrates, which commonly co-occur with these parasites (Orlofske et al. 2012). Specifically, we used Western Mosquitofish (*Gambusia affinis*) and damselfly nymphs (*Enallagma* spp.), both of which consume parasites in laboratory studies (Schotthoefer et al. 2007, Orlofske et al. 2012). Mosquitofish are active, selective foragers with an exceptionally wide diet range (Pyke 2008). Damselflies are largely opportunistic, ambush foragers, with their diet broadly reflecting the prey composition of the habitat (Johnson 1973, Thompson 1978). For our experiments, we selected two body sizes of mosquitofish that roughly correspond to juveniles and adults. The methods for predator collection and maintenance are provided in Appendix 4B and predator body sizes are provided in Appendix 4C.
Table 4.1. Descriptive life history data on the four trematode species used in the predation trials, including body, tail and total length, numbers of individuals measured (*n*), the lighting conditions present during the natural shedding period, and the collection location (USA).

<table>
<thead>
<tr>
<th>Parasite Species</th>
<th>n</th>
<th>Body Length (µm) ± SE</th>
<th>Tail Length (µm) ± SE</th>
<th>Total Length (µm) ±SE</th>
<th>Shedding Period</th>
<th>Collection Location</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Cephalogonimus americanus</em></td>
<td>3</td>
<td>374.1 ±53.8</td>
<td>158.3 ±20.3</td>
<td>532.5 ±73.8</td>
<td>Night</td>
<td>Montana</td>
</tr>
<tr>
<td><em>Echinostoma trivolvis</em></td>
<td>7</td>
<td>343.5 ±18.9</td>
<td>428.4 ±36.8</td>
<td>771.9 ±42.8</td>
<td>Day</td>
<td>Wisconsin</td>
</tr>
<tr>
<td>“Magnacauda”</td>
<td>5</td>
<td>112.7 ±13.9</td>
<td>1066.4 ±104.3</td>
<td>1179.1 ±116.7</td>
<td>Day</td>
<td>Washington</td>
</tr>
<tr>
<td><em>Ribeiroia ondatrae</em></td>
<td>9</td>
<td>426.7 ±33.9</td>
<td>651.2 ±28.1</td>
<td>1077.9 ±56.7</td>
<td>Night</td>
<td>California</td>
</tr>
</tbody>
</table>
Predation Bioassay

We used laboratory bioassays to determine how parasite, predator, and environmental traits influenced the ability of predators to reduce the abundance of free-living cercariae (Orlofske et al. 2012). The experimental design consisted of an individual predator, naïve to parasite prey, provided with a single density of one parasite species under either light or dark conditions. Each individual predator was fasted for 24 hrs prior to assay and used only once. We isolated cercariae from field-collected snails (*Helisoma trivolvis*) by placing them individually in 50-mL centrifuge tubes during their appropriate peak shedding time (day or night). For each assay, we placed a predator in 60 ml of water with 30 cercariae; after 30 min, we removed the predator and counted any remaining cercariae. To examine the effect of light condition on predation, half of the experimental containers were covered with a box sealed to the bench top to prevent all light from entering. Lastly, we explored whether predation by mosquitofish and damselflies was influenced by prey density by examining consumption of *R. ondatrae* and *E. trivolvis* across a range of densities (see Appendix 4D).

Because mosquitofish also have the potential to become infected by some of the parasites used, we necropsied fish 48 hr after trials to quantify encysted parasites (see Appendix 4E for necropsy procedures). We also dissected a subset of damselflies to confirm that they never became infected (*n = 10*/each parasite species) (Schotthoefer et al. 2007). To account for parasite loss in the absence of predators, we also included containers without predators; we successfully recovered all administered cercariae from these control treatments, verifying the effectiveness of our approach in tracking parasites. Although we cannot differentiate failed infection from consumption, we directly observed mosquitofish and damselflies consuming cercariae and recovered a low number of cercariae tails (fish + *R. ondatrae*, 2.9 ± 0.4 [mean ± SE] of 30
parasites administered), which are indicative of attempted infection (Orlofske et al. 2012).

**Statistical Analysis**

We assessed whether predation differed among parasite species, whether this relationship could be explained by parasite or predator size, and how light conditions mediated these relationships. We analyzed the fate of each parasite as consumed or remaining after 30-minutes using generalized linear mixed models with a binomially distributed error and logit-link function in the R package `lme4` (using the Laplace approximation method, R Development Core Team 2008, Zuur et al. 2009). Predictor variables included parasite species, parasite total length (mm), predator body length (mm) and light condition (light or dark). Individual predator identity was included as a random effect to account for variation due to predator satiation or individual differences among predators. We included all possible interactions, which were subsequently reduced if non-significant and models were reassessed (Crawley 2007).

We analyzed data for each predator species separately based on the theoretical and empirical basis that consumption patterns differ between these two predator types (Zaret 1980). For damselflies, we used body length (mm) as a continuous measure of predator size; for fish, we treated body size as a categorical variable (small vs. large) because of the distinct size classes used (see Appendix 4C). Parasite length (mm, tail included) was included as both a linear and non-linear effect in statistical models based on theory derived from zooplankton systems (Zaret 1980).

Depending on the parasite by predator combination, several fates were possible. For damselflies, consumption was equivalent to the number of parasites removed during trials. However, for two of the parasite-by-fish combinations, infection was also possible. For *R.*
Onondatrae, infection of fish can be measured directly by quantifying metacercariae. For Magnacauda where consumption leads to infection (trophic transmission), we considered all the cercariae removed during the trial to be consumed. To examine the effects of predator size and light condition on infection of R. ondatrae and Magnacauda, we used generalized linear mixed models with individual predator identity included as a random effect. For this analysis, we used the number that successfully infected the host vs. not infected (eaten or remaining at the end of 30 mins).

**Results**

**Predation Bioassay**

Damselflies consumed an average of 30% of E. trivolvis and 50% of R. ondatrae cercariae, but <1% of C. americanus or Magnacauda (Z ≤ 2.64, p ≤ 0.008; Figure 4.1A). These differences led to a non-linear relationship between consumption and parasite size (parasite size Z = 11.26, p < 0.0001; (parasite size)^2 Z = −2.97, p = 0.003). The smallest and largest cercariae had the largest influence on the shape of the relationship (Appendix 4F, Table 4.2). Both damselfly size and the absence of ambient light negatively affected parasite consumption (damselfly size Z = −11.34, p < 0.0001; Figure 4.1A; light Z = 3.43, p = 0.0006). Even in the dark, however, there was still a strong non-linear relationship with parasite size (parasite size Z = 14.62, p < 0.0001; (parasite size)^2 Z = −14.74, p < 0.0001), and a negative relationship with damselfly size (Z = −3.73, p < 0.0001; Figure 4.1B). This illustrates the much stronger effect of predator traits relative to environmental condition for these tactile predators (Appendix 4F, Table 4.2).
Table 4.2. Results of generalized linear mixed models examining consumption of parasite prey by two different predator species and the role of parasite species, parasite total length, predator body size, and light condition on those trophic interactions. Parasite species are referenced in table by the first letter of genus and species names (C. a. = *Cephalogonimus americanus*, E. t. = *Echinostoma trivolvis*, R. o. = *Ribeiroia ondatrae*, or Mag = “Magnacuada”).

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<th>P-value*</th>
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<td>0.021</td>
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*Bold = significant at p = 0.05
Mosquitofish were highly effective predators of all parasite species with average consumption ranging from 38 to 75% (Z ≤ 0.73, p ≥ 0.223). Consumption patterns depended on predator size, however (fish size class X parasite size Z = –3.4, p = 0.0006); for all parasites except Magnacauda, small fish consumed more parasites compared to large fish (Z ≤ 7.37, p < 0.0001; Figure 4.2B–D; Magnacauda Z = 1.12, p = 0.262; Figure 4.2A). Light condition had a strongly positive effect on parasite consumption by fish, but only for small fish (light X parasite size Z = –2.23, p = 0.026; light X fish size class Z = 18.05, p < 0.0001, Appendix 4F, Table 4.2). In the dark, large fish generally consumed a greater fraction of parasites relative to small fish (fish size class X parasite size Z = –1.98, p = 0.047). This was true for all parasites except E. trivolvis, for which consumption was equal between predator size classes in the dark (Z ≤ –2.31, p ≤ 0.021; Figure 4.2A, B, and D, E. trivolvis Z = –0.36, p = 0.72; Figure 4.2C).

Infection intensities of Magnacauda in mosquitofish were not related to light condition (Z = 0.16, p = 0.872) or fish size (Z = –1.35, p = 0.179, Figure 4.3A). In contrast, for R. ondatrae, light availability mediated the role of predator size on infection (light x predator size Z = –3.22, p = 0.001; Appendix 4F, Table 4.3, Figure 4.3B). Small fish had lower infection intensities in the light (Z = –3.37, p < 0.001) but not in the dark (Z = –0.27, p = 0.791). The high number of R. ondatrae cercariae eaten by the small fish in the light treatment (Figure 4.2B) suggests that they limited their infection by consuming the parasites before they were able to infect.
Table 4.3. Results of generalized linear mixed models examining infection of mosquitofish by two parasite species under light and dark conditions.

<table>
<thead>
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<th>P-value*</th>
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<td>0.791</td>
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*Bold = significant at p = 0.05
Figure 4.1. Number of parasites eaten by damselfly nymphs under light (gray) or dark (black) conditions across a range of predator body length (4–15 mm) A. Magnacauda B. Ribeiroia ondatrae C. Echinostoma trivolvis D. Cephalogonimus americanus.
Figure 4.2. Mean number of parasites eaten by fish predators under light (open bars) or dark (filled bars) conditions and between two size classes (Large ~30 mm, Small ~10 mm) A. Magnacauda B. *Ribeiroia ondatrae* C. *Echinostoma trivolvis* D. *Cephalogonimus americanus*. Error bars represent ± 1 standard error.
Figure 4.3. Mean number of parasites encysted in fish predators/hosts under light (open bars) or dark (filled bars) conditions and between two size classes (Large ~30 mm, Small ~10 mm) A. *Magnacauda* B. *Ribeiroia ondatrae*. Error bars represent ± 1 standard error.
Discussion

Both predator species were highly effective at consuming free-living parasite stages (50–75% consumption), but this relationship depended critically on predator traits, parasite (prey) traits, and the environmental context in which predator and parasite interacted. Damselflies only consumed parasites of intermediate sizes (0.7–1 mm) and predation declined for larger predators and was slightly reduced in darkness. Fish consumed all parasite species, particularly the largest parasite species (1.2 mm) and small fish were the most effective predators, but darkness strongly reduced their reduced consumption.

Our results indicate that aquatic predators respond to parasites as prey in a manner consistent with patterns based on well-studied relationships between predators and free-living prey (Brooks and Dodson 1965, Zaret 1980). The parasite species we examined spanned a comparable size range relative zooplankton prey (0.2–1.2 mm, Brooks and Dodson 1965). Damselfly predation showed the predicted non-linear relationship with parasite total length, potentially due to reduced detection of *C. americanus*, the smallest parasite, and avoidance of the largest parasite due to handling limitations (Zaret 1980, Hanazato and Yasuno 1989). The negative relationship between consumption and damselfly size could be related to a preference for the largest, manageable prey items, which increases with predator size (Thompson 1978). Predator body size influenced predation more than light availability, which agrees well with predictions based on the ambush foraging mode of these tactile predators (Peckarsky 1982). Predation of parasites by fish was also consistent with consumer-resource theory based on zooplankton. Predation was greatest on the largest parasite (Magnacauda), which rivals the size of large-bodied *Daphnia* prey, although the relationship of consumption was not strictly linear with prey size. This non-linear increase could be related to lower than predicted consumption of
\textit{R. ondatrae}, due to infection reducing cercariae concurrently with predation (Figure 4.3B). Larger bodied fish were less effective predators of cercariae, which is consistent with results from a variety of estuarine fish and cercariae species (Kaplan et al. 2009). As fish grow and mature, the diet shifts towards selection for larger prey, as in mosquitofish with zooplankton (García-Berthou 1999). As visual predators, we also predicted that consumption would be lower in the dark, which was supported with the exception of the largest and hence most visible parasite species (Aksnes et al. 2004).

Predation on free-living parasite stages can lead to important consequences for transmission. For mosquitofish exposed to the directly penetrating parasite, \textit{R. ondatrae}, we observed that small predators limit their own infection in conditions of high visibility. This process may contribute to the low infection levels of \textit{R. ondatrae} in wild mosquitofish populations (Orlofske et al. 2012). Similarly, zebrafish consumption has been observed to limit infection by cecariae of \textit{Trasversotrema patialense} (Anderson et al. 1978). Predation of free-living stages can also reduce transmission to other host species by increasing parasite mortality, sometimes with substantial reductions in host pathology (Hopper et al. 2008, Thielges et al. 2008, Prinz et al. 2009, Orlofske et al. 2012). For the trophically transmitted cercariae, \textit{Magnacauda}, predator traits and light condition were important in determining consumption, but these traits did not influence infection. Low infection intensities suggest that processes independent of initial consumption may have limited infection in mosquitofish.

By incorporating predation of parasites into existing consumer-resource theory developed for freshwater communities, we can make predictions for when predation on parasites may influence transmission and community dynamics natural systems. Based on our findings, both invertebrates and vertebrates are important and predators of parasites. Parasites between 0.5–1
mm are vulnerable to both predator types, while those $\geq 1$ mm are particularly at risk from fish and may be selected to acquire fish as hosts through trophic transmission. The seasonal availability of cercariae may overlap with the presence of small, early ontogenetic stages of predators, which may prefer parasite prey (Kaplan et al. 2009, Preston et al. 2013) and derive significant growth benefits, as also found for certain zooplankton. Intriguingly, parasites that exhibit nocturnal circadian release of free-living stages may improve contact with hosts while decreasing visibility to non-host predators (Lewis et al. 1989, Combes 1991). Previous research with both mosquitofish and damselflies suggests that these predators continue to consume parasites even when alternative prey is available (Orlofske et al. 2012). Our experimental results are potentially applicable for a wide range of parasite densities (Appendix 4D), but a more detailed analysis of functional responses may help predict when predators will regulate parasite populations and therefore transmission (Murdoch and Oaten 1975). Further research should also examine the role of predator-parasite relationships in the stability of aquatic food webs, parasite community dynamics, and evolutionary selection of parasite traits such as circadian rhythms, body size, and transmission mode (Zaret 1980, Lafferty et al. 2008, Johnson et al. 2010).

**Appendices**

*Appendix 4A. Life history information and molecular identification of parasite species.*

Infected snails were collected from field sites in June–August 2009, 2010, and 2011 (Table 4.1). We identified cercariae using a compound microscope to observe distinguishing morphological features from the literature (e.g., Schell 1985, Johnson and McKenzie 2009, Szuroczki and Richardson 2009, Figure 4.4). Samples of cercariae were also vouchered for molecular analysis to verify morphological identification because some species have not yet been described or
cannot be distinguished using morphology alone. Briefly, we obtained genomic DNA of individual cercaria using a Qiagen DNeasy extraction kit and protocol. The internal transcribed spacer region of ribosomal DNA (ITS 1 and ITS 2) gene fragments were independently PCR amplified using GoTaq® Green master mix (Promega, Madison, WI, USA), and the primer pairs: BD1 + 4S (ITS 1) and 3S + ITS2.2 (ITS 2). Protocols for amplification and primer sequence are as described in Bowles and McManus (1993), Bowles et al. (1995), Hugall et al. (1999), and Wilson et al. (2005). Sequencing was performed in both forward and reverse directions using the PCR primers on a Beckman Coulter automated capillary sequencer, and sequence chromatographs were edited using Sequencher 4.2 (Gene Codes Corporation, Ann Arbor, MI, USA). Sequences for each gene fragment were aligned separately, first automatically using the program MUSCLE (Edgar 2004) and, then manually rechecked using Se-Al v2.0a11.

Using these data we were able to identify *Cephalogonimus americanus*, *Echinostoma trivolvis*, and *Ribeiroia ondatrae* to species with 99–100% maximum identity through BLAST searches in GenBank as well as by making Bayesian phylogenetic analyses incorporating the datasets of Razo-Mendivil and Pérez-Ponce de León (2011), Detwiler et al. (2010), and Wilson et al. (2005), respectively. Sequences for “Magnacauda” were not available in GenBank, but our phylogenetic analysis placed it in the family Echinostomatidae. We gathered life history data on all of the species through literature searches (Beaver 1939, Lang 1968, 1969, Dronen and Lang 1974) in addition to direct observation of infection and shedding behavior in the laboratory (Johnson et al. unpublished data). To obtain measurements of cercariae body and tail lengths, we digitally photographed between three and nine unique specimens on a compound microscope (Olympus SZX10) at 10X or 20X magnification. These photos were then analyzed using Image J (Image...
Processing and Analysis in Java, National Institutes of Health) for obtaining cercarial measurements.

Appendix 4B. Laboratory animal collection and maintenance

We collected mosquitofish (*Gambusia affinis*) in June–August 2009, 2010, and 2011 from ponds in central California that were known to be free of the snail host for the trematodes used in our feeding trials. Furthermore, we dissected a subset of fish (n = 10) prior to conducting our experiments to quantify any preexisting infection with the trematode species used in our experiments. Fish were shipped specimens from California to the laboratory at University of Colorado Boulder where we conducted the experiments. Prior to the experiments mosquitofish were maintained in 10-gallon glass aquaria with constant aeration and fed commercial fish food once per day (TetraMin, Tetra®).

Damselflies (*Enallagma* spp.) were collected from two ponds in eastern Colorado. Damselflies are unsuitable hosts for any of the parasites used in our experiments. We maintained damselflies individually in 1-L containers with dechlorinated tap water (AmQuel plus and NovAqua plus, Kordon®) and fed *Daphnia middendorffiana* ad lib.

Appendix 4C. Sizes of species used in predation bioassay and density experiments

Prior to all experiments, body length of each predator was measured to the nearest 0.1 mm with digital calipers and the data are included in Table 4.4.
Figure 4.4. Plate of trematode cercariae used in laboratory study (average total length µm ± SE). A. Magnacauda (1179 ± 117), B. Ribeiroia ondatrae (1077 ± 57), C. Echinostoma trivolvis (771 ± 43), D. Cephalogonimus americanus (532 ± 74, note different scale bar).
Table 4.4. The number \( (n) \) of each predator species examined for the ability to reduce the number of four species of trematode cercariae, and characterize relationship with density with the average body size of the specimens.

<table>
<thead>
<tr>
<th>Parasite Species</th>
<th>Predation Bioassay</th>
<th>Parasite Density Experiment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mosquitofish</td>
<td>Damselfly nymphs</td>
</tr>
<tr>
<td></td>
<td>( n )</td>
<td>( n )</td>
</tr>
<tr>
<td></td>
<td>Body Length (mm) ± SE</td>
<td>Body Length (mm) ± SE</td>
</tr>
<tr>
<td>\textit{Cephalonimus americanus}</td>
<td>10 26.3±0.5</td>
<td>10 9.2±0.1</td>
</tr>
<tr>
<td>\textit{Echinostoma trivolvis}</td>
<td>20 26.0±0.8</td>
<td>20 9.7±0.1</td>
</tr>
<tr>
<td>“Magnacauda”</td>
<td>20 27.2±0.5</td>
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</tr>
<tr>
<td>\textit{Ribeiroia ondatrae}</td>
<td>20 26.1±0.6</td>
<td>20 8.9±0.1</td>
</tr>
<tr>
<td>\textit{Echinostoma trivolvis}</td>
<td>12 11.8±0.3</td>
<td>15 5.6±0.2</td>
</tr>
<tr>
<td>\textit{Ribeiroia ondatrae}</td>
<td>9 10.5±0.2</td>
<td>15 6.4±0.2</td>
</tr>
</tbody>
</table>
Appendix 4D. Parasite density bioassay methods and results

Methods

We used a similar laboratory bioassay to characterize consumption by both mosquitofish and damselfly nymphs to varying densities of two parasite species, *R. ondatrae* and *E. trivolvis*. For each assay, we placed a single individual of each predator species in 60 ml of water with one of three parasite treatments, 30, 60, or 90 cercariae. Trials lasted 30 min, after which we removed the predator and counted any remaining cercariae. Mosquitofish used in these trials were not evaluated for parasite infection because they were euthanized and necropsied within 20 minutes of the trial to evaluate cercariae digestion time (Orlofske et al. *unpublished data*). However, based on the results of the initial bioassay with fish of similar size and light conditions, we assumed that the contribution of infection to the number removed is minimal for *R. ondatrae* and that the fish were refractory to infection with *E. trivolvis*.

We analyzed the relationship of consumption to initial parasite densities for each predator species separately using generalized linear mixed models with a binomially distributed error and logit-link function in the R package lme4 and the Laplace approximation method (R Development Core Team 2008, Zuur et al. 2009).

Results

Consumption of parasites by damselfly predators was positively related to parasite density, but only for *Echinostoma trivolvis* (Table 4.5; Figure 4.5A). Mosquitofish consumption showed a weak relationship with parasite density (Table 4.5). However, the relationship appeared to be negatively related to density for *R. ondatrae* and positive for *E. trivolvis* indicated by a marginally significant interaction between density and parasite species (Table 4.5; Figure 4.5B).
These results suggest that predators continued to consume large proportions of parasites even at relatively high densities and that for the smaller *E. trivolvis* cercariae that predators consumed a higher proportion at higher densities. However, for *R. ondatrae* proportion consumed was either not related to parasite density or showed a negative relationship. This contrast between the two parasite species may be related to size differences between the two parasites, where predators were unable to consume more of the larger parasite during the short (30 min) time trials.

Appendix 4E. Methods for vertebrate and invertebrate necropsy

Mosquitofish were euthanized with buffered MS-222 (Tricaine methanesulfonate, Western Chemical Inc.) and preserved in 10% buffered formalin until necropsy, where we inspected all external surfaces and removed and examined all muscle tissue and organs using a dissecting microscope. Metacercariae were examined under a compound microscope to observe distinguishing features to allow for species identification (Schell et al. 1985, Johnson and McKenzie 2009, Szuroczki and Richardson 2009). Damselflies were dissected following preservation in 70% ethanol. All external and internal surfaces were removed and examined using a dissecting microscope.

Appendix 4F: Supplemental Statistical results for predation and infection

The results of the generalized linear mixed models (GLMM) are presented for the predation bioassays in Table 4.5. Results of the GLMM for infection of *Ribeiroia ondatrae* and “Magnacauda” in mosquitofish are presented in Table 4.5. We assessed variable importance according to BIC values following established criteria (Zuur et al. 2007).
Figure 4.5. Proportion of parasites *Ribeiroia ondatrae* and *Echinostoma trivolvis* eaten at a range of densities for both A. Damselflies and B. Fish.
Table 4.5. Results of generalized linear mixed models of two different predator species and two different parasite species as prey provided at three densities. Data for damselfly predators were analyzed with both parasite species combined, but then subsequently analyzed for each parasite species separately to examine the significant interaction.

<table>
<thead>
<tr>
<th>Predator</th>
<th>Data</th>
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<th>P-value*</th>
<th>BIC</th>
<th>AIC</th>
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<td>0.99</td>
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<td>0.337</td>
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<td>985.1</td>
<td>900</td>
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</tr>
<tr>
<td>Echinostoma</td>
<td>trivolvis</td>
<td>Intercept</td>
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<td>0.155</td>
<td>-0.93</td>
<td>1040</td>
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<td><strong>0.044</strong></td>
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</table>

*Bold = significant at p = 0.05
CHAPTER 5

PREDATION AND DISEASE: UNDERSTANDING THE NET EFFECTS OF AQUATIC PREDATORS ON PATHOGEN TRANSMISSION

Abstract

Predators can have a range of direct and indirect effects on host-parasite interactions, such as direct consumption of infected individuals or infectious parasite stages and changing host behavior and energy allocated to pathogen defense. Because such effects can be either positive or negative, understanding net effects of predation on pathogen transmission is important since in nature predators may play multiple roles. To examine the net effects of predators on interactions between amphibian hosts and their trematode parasites under realistic conditions, we conducted a mesocosm experiment in which we factorially manipulated the occurrence of predators predicted to reduce host behavioral avoidance of parasites (e.g., dragonfly larvae) or reduce the abundance of parasite infectious stages available to infect hosts (e.g., damselfly larvae). The presence of caged dragonflies reduced tadpole activity, resulted in a ~30% increase in infection, and stimulated development of morphological defenses compared with no predators. Rather than reducing infection through consumption of parasites, damselflies elicited behavioral and morphological changes in hosts similar to dragonflies with a comparable increase in transmission. Interestingly, parasite exposure produced phenotypic changes in tadpoles including increased body depth. This research has important implications for understanding the role of biodiversity in disease dynamics within a realistic food web context.
Introduction

Predation is a key process influencing communities and ecosystems through simultaneous effects on prey population dynamics and individual prey traits (Lima 1998, Preisser et al. 2005, Peckarsky et al. 2008). Traditionally, both direct and indirect effects of predators were attributed almost entirely to changes of prey density through lethal interactions (Lima 1998, Peacor and Werner 2001, Werner and Peacor 2003, Preisser et al. 2005, Peckarsky et al. 2008). Direct effects result from physical interactions between two species, while indirect effects require the presence of intermediary species or a set of physical or chemical factors (Abrams et al. 1996). In addition to density-mediated effects, increasing evidence lends support for an equal or even greater role of non-lethal effects of predators, including trait-mediated indirect interactions (TMIs; Werner and Peacor 2003). Trait-mediated indirect interactions in aquatic communities commonly involve kairomones or chemical cues that indicate the presence of the predator, which lead to predator-induced behavioral shifts including changes in foraging activity or habitat use by prey, changes in morphology through phenotypic plasticity, or both (Decaestecker et al. 2002, DeWitt and Langerhans 2003, Werner and Peacor 2003, Benard 2006, Bruno and Cardinale 2008). However, these inducible defenses come at a cost because the expression of a trait may improve performance in an environment with a particular suite of threats but reduce it in another (Decaestecker et al. 2002, Werner and Peacor 2003). Because density and trait-mediated effects occur within communities, it is important to examine both types.

Growing evidence suggests that predation can also have substantial effects on parasitism through a variety of mechanisms (Packer et al. 2003, Ostfeld and Holt 2004, Borer et al. 2009). Predators can reduce the abundance of both susceptible and infected hosts through consumption, thereby reducing transmission (Packer et al. 2003, Ostfeld and Holt 2004). In addition, predators
can consume parasites directly, including free-living infective stages of helminths and ectoparasites, increasing parasite mortality and reducing transmission (Johnson et al. 2010, Johnson and Thieltges 2010). Foraging activity can also result in predators becoming infected through trophic transmission (Lafferty 1999, Hall et al. 2007). Alternatively, predation can increase pathogen transmission indirectly by altering host behavior (Decaestecker et al. 2002). For example, hosts have been shown to reduce activity, including evasive anti-parasite behaviors, in the presence of predators leading to increased infection (Thiemann and Wassersug 2000, Szuroczki and Richardson 2012). Finally, inducible anti-predator defenses may lower host immune responses due to energy allocation trade-offs, therefore increasing parasite transmission (Rigby and Jokela 2000, Navarro et al. 2004, Stoks et al. 2006). As a result of these varied and sometimes conflicting influences, understanding the net effect of predators on host-parasite interactions remains a challenge.

Aquatic communities in general and larval amphibians in particular have served as important model systems for investigating the trait-mediated indirect effects of predators and for understanding host-pathogen interactions (Benard 2004, Koprivnikar et al. 2012). Most studies examining the interactions between predators and parasites of amphibians have focused on a single dimension (Thiemann and Wassersug 2000, Raffel et al. 2010, Szuroczki and Richardson 2012). Belden and Wojdak (2011) illustrate the potential for multiple, simultaneous effects of predators by showing altered trematode infection in larval amphibians through reduced host activity, reduction in host density, or transmission to predators; however, they could not distinguish among individual mechanisms. In addition, empirical studies have verified consumption of free-living infective stages of *Ribeiroia ondatrae*, a pathogenic trematode of amphibians, and further showed that this consumption reduced transmission by 50%.
(Schotthoefer et al. 2007, Orlofske et al. 2012). Some generalist predators could simultaneously influence both parasites and hosts directly through consumption and indirectly due to trait-mediated indirect interactions on behavior and morphology, further emphasizing the need to isolate the multiple roles of predators (Orlofske et al. 2012).

Here, we test the relative importance of two competing effects of aquatic predators on host-parasite interactions: the ability of predators to directly reduce infection by consuming parasite infectious stages before they encounter hosts and the capacity of predators to increase infection by indirectly reducing host anti-parasite behaviors (a trait-mediated indirect interaction). We used semi-realistic mesocosm experiment to represent aquatic communities consisting of Pacific Chorus Frog (*Pseudaris regilla*) larvae as hosts, dragonfly nymphs (*Anax* sp.) as predators of hosts and damselfly nymphs (*Enallagma* sp. and *Lestes* sp.) as predators of the free-living infective stages of the trematode parasite *Ribeiroia ondatrae*. We hypothesized that the presence of caged dragonflies reduces tadpole activity and elicits morphological changes, which potentially increases exposure or reduces energy available to immune defense (Figure 5.1). Based on both of these mechanisms, we predicted higher *R. ondatrae* infection in the presence of tadpole predators. Alternatively, we hypothesized that damselflies directly consume *R. ondatrae* infective stages but do not stimulate trait-mediated indirect effects on host behavior or morphology. We predicted that this would lead to lower *R. ondatrae* infection when damselflies are present (Figure 5.1). By extending our community to include various types of predators, we can integrate both the direct and indirect effects of multiple trophic levels on disease dynamics to better understand how parasite transmission occurs in nature.
Figure 5.1. Conceptual diagram illustrating the hypothesized direct and indirect effects of predators on parasite transmission and host pathology. Damselfly nymphs may directly consume (solid black line) *Ribeiroia ondatrae* free-living infective stages leading to lower density of parasites to potentially transmit (solid gray line) to tadpole hosts. Damselflies may also stimulate indirect effects (dashed line) on tadpole behavior and morphology. Dragonfly nymphs may cause indirect effects (dashed line) on tadpole morphology and behavior. Parasites may also alter host behavior and morphology through indirect effects (dashed line). Increased pathology (white arrow) may result from increased infection due to indirect effects of predators. Width of the arrows indicates the strength of the effects (narrow = weak, wide = strong. (Note: Dragonflies were prevented from direct consumption of tadpoles in our experiment and therefore that interaction was not included in this diagram).
**Methods**

*Study system*

*Ribeiroia ondatrae* is a trematode with a complex life cycle, sequentially infecting first intermediate host snails (*Helisoma trivolvis*), second intermediate host amphibians, and finally amphibian-eating birds as definitive hosts (Johnson et al. 2004). Transmission of *R. ondatrae* from snail to amphibians occurs through direct infection by free-living aquatic parasite stages known as cercariae, which then form encysted metacercariae in the amphibian (Johnson et al. 2004). Mortality and pathology in amphibians is based on the intensity of infection (i.e., total number of parasites in the host) highlighting the need for understanding the role of the broader aquatic community in transmission dynamics (Johnson et al. 1999). The Pacific Chorus Frog, *Pseudacris regilla*, serves as our focal amphibian host because it is among the species with the highest reported frequencies of malformations, approaching 90% among newly metamorphosed (juvenile) frogs at some wetlands (Johnson et al. 1999, 2001, 2002). In addition, this species expresses inducible defenses (e.g., reduced activity level, deeper tails) in response to aquatic predators (Benard 2006).

We created biologically relevant aquatic predator communities using damselflies and dragonflies, which are highly abundant and frequently co-occur in wetland environments supporting both intermediate host snails of *R. ondatrae* and Pacific Chorus Frogs (Orlofske et al. 2012, Preston et al. 2013). We selected damselfly nymphs (*Enallagma* sp. and *Lestes* sp.) as predators of *R. ondatrae* cercariae because previous laboratory studies have shown that they actively consume cercariae even in the presence of alternative food sources and can reduce transmission in small-scale laboratory experiments (Schotthoefer et al. 2007, Orlofske et al. 2012). We chose dragonfly nymphs (*Anax* sp.) as our tadpole predators based on the well studied
interactions between amphibian larvae and dragonflies, including reduced host behavior, changes in tadpole tail and body morphology, and corresponding increases in parasite infection intensity (Thiemann and Wassersug 2000, Relyea 2004). Furthermore, late instar *Anax* sp. do not consume parasites and neither dragonflies or damselflies function as hosts, allowing us to isolate the role of trait-mediated indirect interactions (Orlofske et al. 2012). Although dragonflies also serve as predators of damselflies, previous research has indicated that presence of caged dragonflies did not influence damselfly feeding rate, which is the primary mechanism by which we expect damselflies to influence parasite infection (Stoks et al. 1999, Stoks 2001).

*Mesocosm establishment and maintenance*

We established 32 mesocosms using 68-L tubs (Rubbermaid) within an experimental greenhouse following standard methods (Johnson et al. 2012; Appendix 5A). On 14 June 2011, mesocosms were filled with 5.7 kg of sand and 50 L of tap water. After 1 week, we added four grams of crushed rabbit chow, 0.015 L of pond sediment, and 0.4 L of filtered pond water collected from local wetlands. Finally, we established zooplankton communities with a total of 0.095 L of concentrated zooplankton from local wetlands and added a 20 cm length of polypropylene rope, simulating aquatic vegetation, to provide added substrate for tadpoles and damselflies (Michel and Burke 2011). We continuously monitored water temperature in a subset of mesocosms throughout the experiment (*n* = 14; Hobo underwater datalogger, Onset Computer Corporation, Bourne, MA; Appendix 5A).

We began the experiment 02 July 2011 by adding ten Pacific chorus frog tadpoles at stages 28 or 29 (Gosner 1960) and average mass 0.180 mg ± 0.005 standard error (SE) to each mesocosm that had been collected as eggs and maintained in the laboratory (Appendix 5B). We
randomly assigned mesocosms to a treatment condition based on a 2 X 2 X 2 factorial design with the presence/absence of *Ribeiroia ondatrae* cercariae, presence/absence of damselflies, and presence/absence of caged dragonflies. Each treatment was replicated four times. To each mesocosm assigned damselflies, we added ten nymphs randomly selected from a mixture including *Enallagma* sp. and *Lestes* sp. collected from local wetlands (average body length 14.0 mm ± 1 SE; Appendix 5B). Because we were interested in examining the role of non-lethal predator effects, we maintained dragonflies in cages throughout our experiments. For mesocosms assigned to a caged predator treatment, we placed a single *Anax* sp. dragonfly nymph collected from local wetlands (average body length 41.2 mm ± 1.2 SE) in a 0.24-L plastic cup covered with a piece of plastic window screen to prevent escape (Appendix 5B). Dragonflies were fed a comparably sized conspecific tadpole as the experimental tadpoles prior to being added to the mesocosms and were fed similarly every three days (tadpole mass range 0.180-0.600 mg). If a dragonfly did not eat the tadpole within 12 hours it was replaced with an extra dragonfly that had eaten in the laboratory (see Appendix 5B). Mesocosms that did not receive a dragonfly predator contained an empty 0.24-L plastic cup cage and were taken out and replaced when dragonflies were fed to control for any effect of disturbance. We conducted daily censuses of dragonflies and damselflies in the mesocosms. We replaced dragonflies or damselflies if they were found dead or emerged from the mesocosms indicated by the presence of exuviae along the edges of the mesocosm.

*Parasite exposure*

For all experimental procedures, field-collected *R. ondatrae* infected snails were placed in 50-mL centrifuge tubes from 18:00 – 22:00H to collect newly emerged cercariae. Cercariae were
counted using a glass pipette under a dissecting microscope and transferred to 50-mL centrifuge tubes before being added to the mesocosms. We added cercariae to mesocosms at midnight in accordance with when they would be available in nature (Johnson et al. 2004). Mesocosms in no parasite treatments received 35 mL of snail-conditioned water as a sham exposure. Immediately prior to adding the parasites, we ensured that no uneaten tadpoles were present in the predator cups that could potentially become infected and reduce transmission to the experimental tadpoles. We added a total of 400 cercariae to each mesocosm with additions of 50 cercariae on July 2 and 3 and 100 cercariae on July 6, 9, and 11. We chose the level and duration of exposure to balance identifying differences in transmission dynamics due to the hypothesized mechanisms, while minimizing the lethal effects of *R. ondatrae* on tadpoles.

**Amphibian behavior**

We quantified tadpole activity (the number of active tadpoles defined as any movement of the tadpole through the water) using scan sampling (Michel and Burke 2011, Szuroczki and Richardson 2012). Because of the small mesocosm sizes and limited structural complexity, all tadpoles were visible during observations. Each mesocosm was observed 10 times and average number of tadpoles active was used for each mesocosm in subsequent analyses. These observations were performed once (1200-1300H) each day from 07 to 13 July 2011.

**Amphibian growth and morphology**

We ended the experiment 14 July 2011 so that we could assess phenotypic plasticity in tadpole traits prior to metamorphosis. All tadpoles were euthanized in MS-222 (Tricaine methanesulfate,
Western Chemical Inc.) buffered with sodium bicarbonate, weighed to the nearest 0.001 mg, and preserved in 10% buffered formalin. Tadpoles were staged according to Gosner (1960).

To assess phenotypic plasticity, we photographed each tadpole on the right side with a glass slide under the tail in identical positions using a dissecting microscope (Olympus SZX10) and digital camera (Olympus Corporation) with a 1 cm scale bar. We characterized tadpole morphology based on 7 linear measurements (total length = TOL, tail length = TL, tail depth = TD, tail muscle depth = MD, body length = BL, body depth = BD, and mouth width = MW) using Image Processing and Analysis in Java software (ImageJ, National Institutes of Health; Relyea 2001, Appendix 5C).

**Amphibian malformation and parasite infection**

To evaluate pathology, we examined every tadpole under a dissecting scope for the presence and type of any malformations. To quantify *R. ondatrae* transmission success we randomly selected 5 of the 10 total tadpoles in each mesocosm to quantify parasite infection intensity (Appendix 5D). In addition, we dissected a random subset of 1-2 tadpoles per mesocosm that were not in parasite treatments to verify that they were never exposed.

**Zooplankton abundance**

We quantified zooplankton density at the end of the experiment based on samples collected with a tube sampler (30 cm in length x 5 cm in diameter, two combined samples per mesocosm) and passing them through a 45-µm sieve. Samples were preserved in 70% ethanol and later identified and counted under a dissecting microscope.
Statistical Analysis

We examined *R. ondatrae* infection intensity using a linear mixed effects (LME) model with mesocosm as a random effect in the R package nlme (R development Core Team 2008); however, our necropsies verified that no tadpoles from unexposed treatments were infected so our analyses included only mesocosms exposed to *R. ondatrae*. To examine pathology, we scored each tadpole as malformed or normal. We used generalized linear mixed models in the R package lme4 with the Laplace approximation method, binomially distributed error and logit-link function to test for effects of predators with mesocosms included as a random effect (Zuur et al. 2009). Because we never observed malformations in mesocosms unexposed to parasites, we only analyzed malformations from mesocosms exposed to *R. ondatrae*. We used repeated measures analysis of variance (rm-ANOVA) to test the effects of different communities on tadpole behavior over the course of the experiment (JMP Pro 9). Similar to the infection analysis, we evaluated the role of different species on tadpole mass and developmental stage using LME with mesocosm identity as a random effect. To compare zooplankton communities across treatments, we used a LME on log transformed total zooplankton abundance with mesocosm as a random effect. Non-significant interactions were sequentially removed from the final models. Tadpoles from one mesocosm with a no predator or parasite treatment exhibited unusually slow development and small wet mass due to an overgrowth of blue-green algae and were removed from all analyses.

To analyze differences in phenotypic plasticity across parasite and predator treatments, we first needed to remove differences that were due to the allometric relationship between morphological traits and tadpole size (Hoverman and Relyea 2012). To address size variation, we used analysis of covariance (ANCOVA) with log_{10} transformed mass as the covariate.
(Hoverman et al. 2005). A critical assumption of the ANCOVA is a common slope of the regression across treatments (i.e. similar allometric relationships). We identified three traits that met this assumption (tail depth, tail muscle depth, and body depth) and used those for further analysis. We used the mass-adjusted treatment mean and residuals from the within-treatment regression to calculate each individual’s size-adjusted trait value. Then we calculated the mean response for each experimental unit within each sample and used these as our morphological response variables. Because these measurements represent multiple responses from the same individuals, we used multivariate analysis of variance (MANOVA), followed by univariate analyses (ANOVA) for each trait that was significant in the previous analysis.

**Results**

*Amphibian malformation and parasite infection*

The presence of either dragonflies or damselflies caused an increase in amphibian infections by *Ribeiroia ondatrae* relative to treatments without predators (LME; Dragonfly: $t_{12} = 2.5, p = 0.029$; Damselfly: $t_{12} = 2.4, p = 0.035$; Dragonfly X Damselfly: $t_{12} = -1.8, p = 0.102$; Figure 5.2). While there was no interaction between the predators, hosts maintained with either or both predators supported, on average, 52 to 54% more parasites than those with no predator present. Only two tadpoles died during the experiment and neither were exposed to parasites, verifying our sub-lethal exposure levels and reducing any influence of density-dependent changes in transmission. Malformations were only detected in the region of the developing hind limb buds and only in mesocosms exposed to *R. ondatrae*, as expected. Types of malformations varied from twisted or bent limbs, extra digits, to extra limb buds. The proportion of malformations
Figure 5.2. Average infection of Pacific Chorus Frog (Pseudacris regilla) tadpoles from mesocosms (n = 4/treatment) exposed to Ribeiroia ondatrae in the absence (control) or presence of caged dragonflies, unrestrained damselflies, or both species combined. Error bars represent ± 1 standard error.
varied from 10 to 28% but did not differ significantly as a function of predator treatment (GLMM; $Z > -1.23$, $p > 0.22$).

**Amphibian behavior**

Predator treatment, time, and the time-by-dragonfly interaction all significantly affected tadpole behavior during the experiment (see Appendix 5E, Table 5.1). The presence of caged dragonflies reduced tadpole activity, and this reduction was most pronounced (~20% decrease relative to no-predator treatment) during the first three days of observations (ANOVA; Dragonfly X Time, $F_{6,19} = 12$, $p < 0.0001$, Figure 5.3A). Likewise, damselflies also significantly reduced tadpole activity over the entire experimental period (ANOVA; $F_{1,24} = 7.8$, $p = 0.010$, Figure 5.3B). However, the tadpoles only reduced their activity by 13% in response to the damselflies. Surprisingly, tadpoles exposed to *R. ondatrae* did not show any differences in activity (ANOVA; $F_{1,24} = 1.5$, $p = 0.230$). In general, the percentage activity of tadpoles increased over time (ANOVA; $F_{6,19} = 25.6$, $p < 0.0001$).

**Amphibian growth and morphology**

Both dragonflies and parasites influenced phenotypic changes in tadpole morphology (MANOVA; Dragonflies: $F_{3,21} = 42.4$, $p < 0.0001$, Parasite: $F_{3,21} = 4.2$, $p = 0.039$; interactions: $F_{3,21} < 2.6$, $p > 0.08$, Figure 5.4A-D). Effects of damselflies on tadpole morphology were only significant when examined for individual traits. The presence of either predator resulted in a significant increase in tadpole tail depth (ANOVA; Dragonflies: $F_{1,23} = 109.8$, $p < 0.0001$, Damselflies: $F_{1,23} = 5.4$, $p = 0.029$, Parasite: $F_{1,23} = 7.5$, $p = 0.012$; Figure 5.4E). Similarly,
Table 5.1. Results of repeated-measures ANOVA on the effects of caged dragonfly predators, unrestrained damselflies, and *Ribeiroia ondatrae* parasites on tadpole activity over time. Significant p-values are indicated in bold.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>DF</th>
<th>F</th>
<th>p</th>
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</thead>
<tbody>
<tr>
<td><strong>Between Subjects</strong></td>
<td></td>
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<tr>
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<tr>
<td><strong>Within Subjects</strong></td>
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<tr>
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Figure 5.3. Proportion of active Pacific Chorus frog (*Pseudacris regilla*) tadpoles (*n* = 10) based on daily snap-shot observations of individual aquatic mesocosms (*n* = 4/treatment) A. in the presence or absence of caged dragonfly nymphs fed conspecific tadpoles or B. in the presence or absence of 10 unrestrained damselfly nymphs. Predators were present prior to and throughout the days behavior was monitored while parasites were added at midnight indicated by the black arrows. Error bars represent ± 1 standard error.
Figure 5.4. Images of representative Pacific Chorus Frog (*Pseudacris regilla*) tadpoles from mesocosms with factorial combinations of caged dragonflies, unrestrained damselflies, and the trematode parasite *Ribeiroia ondatrae*: A. No predators or parasites present; B. Dragonflies only present; C. Parasite only present; D. All species (dragonflies, damselflies, and parasites) present. Scale bar = 1 cm. Interaction plots of tadpole morphological data adjusted for tadpole mass and averaged across mesocosms: E. Tail muscle depth by dragonfly and damselfly absence (N) or presence (Y); F. Body depth by dragonfly and parasite absence (N) or presence (Y). Error bars represent ± 1 standard error.
tadpoles in the communities with dragonflies (ANOVA; \( F_{1,23} = 53.5, p < 0.0001 \)) or damselflies (ANOVA; \( F_{1,23} = 4.7, p = 0.041 \)) had deeper tail muscles. Both parasites and dragonfly presence were associated with deeper tadpole bodies (ANOVA; Dragonfly: \( F_{1,23} = 16.0, p = 0.0005 \), Parasite: \( F_{1,23} = 7.0, p = 0.014 \)). However, the effect of parasites was more pronounced without dragonflies present (ANOVA; Dragonfly X Parasite: \( F_{1,23} = 4.4, p = 0.047 \); Figure 5.4F). Neither predators, parasites, nor their interactions had any significant effects on tadpole mass (LME; \( t_{28} = 1.59, p > 0.123 \)) or developmental stage (LME; \( t_{28} = -1.37, p > 0.183 \)). Across all treatments, tadpole wet mass increased to 0.824 mg \( \pm \) 0.011 SE and developmental stage increased to 36.1 \( \pm \) 0.1 SE (Gosner 1960).

**Zooplankton abundance**

*Daphnia middendorffiana* made up over 97% of the zooplankton community in our mesocosm with copepods composing the rest of the community. Zooplankton abundance across the pooled samples was 338 \( \pm \) 70 per mesocosm and did not differ significantly across predator or parasite treatments (LME; \( t_{27} > 0.83, p > 0.41 \)).

**Discussion**

Our results indicate that multiple trophic levels in aquatic ecosystems can alter pathogen transmission through trait mediated indirect interactions with hosts. Using a mesocosm experiment, we examined predator-parasite interactions in a community that better represents natural ecosystems (Bruno and Cardinale 2008, Spivak et al. 2011). In contrast to previous research, we were able to isolate potential mechanisms by using invertebrate predators that do not simultaneously serve as hosts to the parasite (Raffel et al. 2010, Belden and Wojdak 2011).
In addition, we exposed tadpoles to sub-lethal levels of parasites to reduce the role of density-dependence in the host population but still at biologically relevant levels capable of inducing pathology (Belden and Wojdak 2011, Johnson et al. 2013). Overall, we observed an increase in *R. ondratrace* infection in amphibian hosts in the presence of dragonflies, damselflies and both species combined. The potential mechanisms for the increase include trait-mediated indirect interactions of both predators on tadpole behavior and morphology. Surprisingly, parasite exposure and infection also elicited morphological changes but did not alter host activity.

Consistent with our predictions, the presence of caged dragonflies resulted in higher *R. ondratrace* transmission, reduced host activity and development of inducible anti-predator defenses. Other studies found that for amphibians that are vulnerable to predators infection increases in their presence (Thiemann and Wassersug 2000, Szuroczki and Richardson 2012). Conversely, predation risk did not affect *Echinostoma trivolvis* infections in larval amphibian hosts in a mesocosm experiment suggesting that there may be important life history trade offs through development rate and other characteristics of the host-pathogen system (Raffel et al. 2010). However, in their experiment tadpoles were exposed directly to infected snails, which may also contribute to differences between the two studies.

We identified reduced activity, including anti-parasite behavior, as the primary mechanism for the increase in infection caused by predators (Thiemann and Wassersug 2000). Indeed, anesthesia of tadpoles, which simulates the reduced activity due to predation, results in higher infection compared to active tadpoles (Koprivnikar et al. 2006, Daly and Johnson 2011, Chapter 2). In the presence of dragonflies, tadpoles developed deeper bodies and deeper tails, which have been well characterized in pervious studies (Benard 2004, Relyea 2004). This suggests a second potential mechanism for increased infection involving reduced investment into
host immune responses due to resource allocation into inducible defenses or stress induced
immune suppression (Rigby and Jokela 2000, Navarro et al. 2004, Stoks et al. 2006, Middlemis
et al. 2013). We did not measure components of the immune system; however, a similar
mesocosm study using non-lethal predation cues did not find a relationship between predators
and tadpole immune responses (Raffel et al. 2010). In this system behavioral and morphological
traits were used in a compensatory manner (DeWitt et al. 1999, Rundle and Brönnmark 2001,
DeWitt and Langerhans 2003). Tadpoles exposed to dragonflies reduced activity more
dramatically early in the experiment potentially as morphological responses developed allowing
the tadpoles to increase activity later on in the experiment. Similarly, more vulnerable life stages
of crayfish exhibit a greater degree of behavioral response to predatory fishes (Keller and Moore
1999). As prior studies have shown, we found no effects of non-lethal dragonfly cues on tadpole
growth rate or development (Theimann and Wassersug 2000, Benard 2004).

In contrast to our predictions, the presence of damselflies also elicited anti-predator
responses and subsequent increases in transmission to amphibian hosts. Based on laboratory
studies showing reduced transmission due to consumption, we predicted lower infection in
damselfly treatments (Orlofske et al. 2012). The observed differences may be due to some
important features of our more realistic mesocosm experiment. For example, alternative prey
could have reduced damselfly predation on cercariae since we had a thriving zooplankton
community in our mesocosms. However, the presence of damselflies did not affect zooplankton
abundance and previous laboratory experiments indicated that damselflies continued to consume
parasites in the presence of alternative prey (Orlofske et al. 2012). Another potential factor could
be reduced consumption rate of parasites by damselflies in darkness. As visual predators,
damselflies may not be as effective at night when we added parasites based on natural shedding
patterns of *R. ondatrae* (Chapter 4). In addition, damselflys’ preference for cercariae prey may change with predator body size (Chapter 4). Given that damselflies were developing over the course of the experiment, we maintained equal damselfly densities by replacing those that metamorphosed. Furthermore, damselflies continue feeding until late metamorphosis when the labium atrophies so that the amount of time they were not feeding prior to emergence would have been minimal (Lawton 1970).

Damselfly presence had a similar influence on transmission to that caused by the presence of dragonflies, corresponding with changes in tadpole activity and morphology. Importantly, we observed that morphological changes were in the same direction between the two taxa of odonates but stronger for the dragonflies because they are the more risky predator (DeWitt et al. 2000). This effect may be due to similarities of the chemical cues eliciting the response and the functional basis for predator-induced plasticity that confers protection (Van Buskirk and Relyea 1998, Theimann and Wassersug 2000). Despite the trait-mediated indirect interactions observed, we suggest that damselflies did not cause malformations though attempted predation on tadpoles, because malformations were never observed in the absence of *R. ondatrae* infection (Ballengée and Sessions 2009, Bowerman et al. 2010).

Our results indicate that parasites alter aspects of their host morphology to a similar extent as predators but did not influence overall activity. The parasite exposure level and timing we used was successful at producing non-lethal infections but at the same time resulted in over one quarter of exposed tadpoles with detectable limb malformations (Johnson et al. 2011). More surprisingly, we observed changes in tadpole morphology, including body depth, in the presence of parasite infection. These changes may have been reflective of a general stress response, induced to help the tadpoles remove cercariae, or a direct response of the tadpole to the parasites
that frequently encyst in the area of the developing limb buds and along the base of the tail. The increase in body depth due to parasite exposure was similar to that induced by dragonflies suggesting that there were no trade-offs in the responses to different natural enemies (Raffel et al. 2008). Hosts may engage in anti-parasite behavior prior to infection or infection can directly influence activity (Lefcort and Eiger 1993, Levri 1999, Rohr et al. 2009, Szuroczki and Richardson 2012). We did not observe any changes in tadpole activity in response to parasites; however, exposure took place at night and our observations took place during the day. Therefore, parasite evasion or temporary reduction in activity with infection may have not been detected (Szuroczki and Richardson 2012). Future research should investigate the functional significance of host behavior and morphological responses to parasites in the presence of other natural enemies as one step towards integrating parasites into natural enemy ecology (Raffel et al. 2008).

Overall, our research demonstrates the importance of examining multiple trophic levels, their interactions, and underlying mechanisms to further develop a community ecology framework for disease dynamics in natural systems. Previous studies have examined the transmission consequences of the direct effects of predators reducing parasite and host abundance individually (Packer et al. 2003, Ostfeld and Holt 2004, Borer et al. 2009, Orlofske et al. 2012). In addition, trait-mediated indirect interactions including altered habitat selection, activity levels, and physiological responses can have significant impacts on infection (Rigby and Jokela 2000, Decaestecker et al. 2002, Raffel et al. 2010). In order to fully integrate predation and disease dynamics, trait-mediated indirect interactions need to be considered simultaneously with direct effects on densities of hosts and parasites. By isolating potential mechanisms, we found that indirect effects of predators on hosts resulting in increased contracts with infective stages or increased susceptibility to infection may be more important than direct effects of
parasite consumption. Together, this may mean that the presence of non-host species in the community may increase infection in target hosts despite potential negative effects on parasite abundance.

An emerging area of biodiversity research focuses on the role of predation in ecosystem processes and functions, including pathogen transmission (Bruno and Cardinale 2008, Raffel et al. 2010, Belden and Wojdak 2011). This research is essential given rapidly changing biodiversity and coinciding disease emergence (Daszak et al. 2000). Specifically, predators tend to have a disproportionately high probability of extinction, while at the same time they are frequently introduced (Davies et al. 2000, Olden et al. 2004, Dobson et al. 2006, Strayer 2010). Invasive predators may have both direct and trait-mediated indirect interactions with native species (Pujol-Buxó et al. 2012). An important next step is to use gradients in species diversity and abundance to evaluate net effects of predators on pathogen transmission in the field.

**Appendices**

*Appendix 5A. Additional mesocosm methods*

Given that the small water volume of the mesocosms would make them sensitive to temperature shifts, it was unfeasible to place the mesocosms outside. Therefore, we established our mesocosms in the campus greenhouse facilities (University of Colorado Boulder campus, Boulder, Colorado, USA) to maintain a semi-realistic setting, but protect them from rapid and extreme temperature fluctuations that may result in mortality of study species. Light conditions corresponded with natural patterns. Throughout the experiment, we added two liters of aged tap water to all tanks every other day to offset evaporation. We established zooplankton communities over six days, though a series of three additions (0.04, 0.02, and 0.035 L) of
concentrated zooplankton from local wetlands. We did not test the species of zooplankton in our mesocosms to see if they had any negative or positive effects on parasite transmission. However, laboratory tests with another *Daphnia* species indicated that they do not consume cercariae of *R. ondatrae* (Schotthoefer et al. 2007).

We randomly assigned temperature loggers to treatments located throughout the mesocosm array and placed them in the center of 14 mesocosms, 10 cm from the bottom, to monitor temperature hourly over the course of the experiment. The average temperature in the mesocosms was $23.3 \pm 0.4^\circ C$ over the course of the experiment.

**Appendix 5B. Animal collection and maintenance**

We obtained Pacific Chorus Frog (*Pseudacris regilla*) eggs from field sites in California, USA. We maintained tadpoles in 40-L plastic containers with airstones and fed a 1:1 mixture of commercial fish food (TetraMin and Spirulina) with 50 percent water changes occurring every 2–3 days until tadpoles reached approximately stage 28 or 29 (Gosner 1960). We maintained 100 tadpoles under these conditions in the laboratory for the duration of the mesocosm experiment to feed to the caged dragonflies (see below).

Snail first intermediate hosts (*Helisoma trivolvis*) naturally infected with *Ribeiroia ondatrae* were collected from field sites surrounding San Francisco Bay, California, USA (Contra Costa, Alameda, Santa Clara and San Mateo counties).

Damselflies and dragonflies were collected from natural wetlands in Boulder, Colorado, USA. Dragonflies were maintained individually in 1-L containers and fed a Pacific Chorus Frog tadpole every three days and immediately before being placed in the mesocosm. In addition, we maintained twelve extra dragonflies in the laboratory and fed tadpoles on the same schedule as
the mesocosm experiment so that they could replace any dragonflies that died or refused to eat when approaching metamorphosis. Damselflies were pooled across wetlands and randomly assigned to mesocosms. Subsamples were preserved in 70% ethanol for identification. Finally, we maintained extra damselflies in the laboratory on cultures of *Daphnia middendorffiana* and used them to replace individuals that were found dead or metamorphosed.

*Appendix 5C. Landmarks for amphibian morphological assessment*

We choose seven linear dimensions to evaluate phenotypic plasticity of tadpoles based on Relyea (2001), including total length, tail length, tail depth, tail muscle depth, body depth, body length, and mouth size (Figure 5.5).
Figure 5.5. Digital image of preserved Pacific Chorus Frog (*Pseudacris regilla*) tadpole illustrating the positions of the morphological measurements taken to evaluate phenotypic plasticity including total length (red), tail length (orange), tail depth (yellow), tail muscle depth (green), body depth (light blue), body length (dark blue), and mouth size (purple).
Appendix 5D. Methods for amphibian necropsy

For each necropsy, we inspected all external surfaces and removed all muscle tissue and organs and examined them using a dissecting microscope. We examined metacercariae under a compound microscope to observe distinguishing features to allow for species identification (Johnson and McKenzie 2009, Szuroczki and Richardson 2009).

Appendix 5E. Statistical results of tadpole behavior

Here we provide the table of all the within and between subjects comparisons, including the 2-, 3-, and 4-way interactions of the repeated measures analysis of variance for tadpole activity (Table 5.1).
CONCLUSION

Understanding the role of community ecology in disease dynamics is becoming increasingly important in light of biodiversity change through species and population extirpations and introductions of non-native species worldwide (Daszak et al. 2001, Pongsiri et al. 2009, Keesing et al. 2010). Increasing evidence suggests that parasite-host interactions occur within a wider community with the potential to influence transmission dynamics and pathology (Keesing et al. 2006, 2010). The main objective of my dissertation was uniting predator and parasite-host interactions to improve our understanding disease dynamics in natural communities. My dissertation consisted of four main components. In the first, I sought to characterize transmission of macroparasites as a prerequisite for understanding how infection patterns were altered in more diverse systems. In the second and third, I examined the potential for aquatic predators to alter parasite transmission through consumption of free-living parasite stages and how predator-mediated changes to transmission varied with environmental condition and parasite species. Finally, I investigated the net effects of predation including both direct consumption of parasites and trait-mediated indirect effects on hosts. Overall, my dissertation research contributes both to a more mechanistic understanding of macroparasite transmission dynamics and to the direct and indirect roles of ecological communities in mediating host-parasite interactions. In addition, my research demonstrates the parallels between host-pathogen and predator-prey interactions providing important conceptual integration between these two fields of ecology. These results can serve as a foundation for future research on the community ecology of disease for *Ribeiroia ondatrae* and other host-pathogen systems. Extensions of this research include modeling the effect of spatial scale on parasite transmission dynamics and aggregation within and among
hosts, characterizing the functional response of predator consumption to varying densities of parasite prey, and investigating the patterns of predator diversity and abundance with infection prevalence and intensity in nature. Below, I discuss in more detail how my research has improved our understanding of *R. ondratae* transmission, suggest directions for future research, and describe the implications for biodiversity change and disease ecology.

*Transmission dynamics of macroparasites in a community context*

By building from identifying the functional form of transmission and evaluating predator-parasite interactions to more realistic communities, I described more fully how species interactions may shape *R. ondratae* transmission dynamics. Through my laboratory experiments with *R. ondratae* and *Pseudacris regilla* tadpoles, I identified non-linear functions as the best phenomenological models for representing transmission. Knowledge of these functional forms is not only crucial for understanding the community interactions that may influence *R. ondratae* infection in nature, but also provides a foundation for future studies of transmission in other host-pathogen systems, suggests potential mechanisms for non-linear patterns, and highlights similarities with other consumer-resource interactions. Previous theoretical analyses of non-linear transmission functions suggest that there is a threshold density of infective stages required for disease persistence, in contrast to only a threshold of susceptible hosts (Gubbins and Gilligan 1997). Therefore, other species in the community with the potential to deplete the number of infective stages, such as alternative hosts or predators, may influence transmission dynamics in nature (Civitello et al. 2013).

One hypothesis for how community diversity influences disease dynamics is called the “dilution effect” and proposes that more diverse communities lead to reduced transmission
Most studies of the dilution effect tend to focus on alternative hosts that differ in their abilities to transmit the pathogen (Keesing et al. 2006, 2010). My dissertation extends the previous focus of dilution effect mechanisms from alternative hosts to the potential role of predators of parasites. By examining the members of aquatic communities co-occurring with *R. ondatrae* based on field data, I showed that several species consume cercariae independently of other mechanisms of the dilution effect, such as serving as alternative hosts. Taking my study one step further, I showed that consumption by damselfly nymphs reduced transmission of *R. ondatrae* to Pacific chorus frog tadpoles. Transmission was reduced to similar extent when newts were present as alternative hosts, suggesting that predation can influence infection to the same extent as alternative hosts. In support of our experimental results, necropsy data from naturally infected hosts confirmed that newts are commonly infected to the same intensity as co-occurring *P. regilla* tadpoles.

To evaluate how predation of parasites varies with species traits and environmental conditions, I examined the consumption of multiple parasite species by both fish and insect predators in light and dark conditions. I found that small-bodied predators were the most effective at reducing numbers of cercariae. However, predators differed in their feeding abilities based on gape-limitations and light availability. Fish predation was highest on the largest parasite species, whereas damselflies consumed intermediately sized parasite species the most. Although damselflies were less effective predators in the dark, the role of parasite and predator body size was much stronger. In contrast, in dark environments, the role of body size in fish predators was switched with larger fish consuming more parasites than smaller fish. Then I compared my results for parasites with theory developed for predation on zooplankton, which may provide a
predictive framework for understanding the role of predation on parasite transmission using a trait-based approach (Brooks and Dodson 1965, Zaret 1980).

In natural communities, predators of hosts are also capable of influencing transmission dynamics through a variety of mechanisms, including reduction of infected and susceptible host densities or alteration of host behavior (Decaestecker et al. 2002, Packer et al. 2003). In the final chapter of my thesis, I sought to examine the net effects of predator communities on parasite transmission by comparing the relative strength of direct consumption of parasites by predators to trait-mediated indirect effects elicited by predators of hosts. To do so, I used a semi-realistic mesocosm experiment with factorial combinations of parasites, predators of parasites, and predators of hosts. I found that both types of predators increased R. ondratae infection in P. regilla tadpoles. Potential mechanisms underlying higher infections include trait-mediated indirect effects on host activity and energy allocation to developing morphological defenses. This suggests that these indirect effects may outweigh the direct effect of reduced parasite abundance through consumption in mediating transmission. Importantly, these results show that it may be the specific role of the species in the community rather than biodiversity per se that is important for influencing transmission dynamics in nature.

My dissertation research integrates predator and parasite ecology considering both the direct roles of consumption of parasites by aquatic predators and the trait-mediated indirect effects of predators on hosts. By examining a suite of transmission functions, I was able to find parallels between parasite transmission, parasitoid searching behavior, and functional responses of predators providing further evidence for a united theory of natural enemy ecology (Raffel et al. 2008). My results also emphasize the diversity of functional roles of predators within the aquatic communities where parasite transmission occurs. Many predators reduce the abundance
of cercariae in the laboratory even in the presence of alternative prey but whether this consumption leads to reduced transmission in nature may depend on traits of particular species and developmental stages. Because predators at early stages of development consume more parasites, we may expect that parasite transmission may be influenced not only by the diversity and abundance of particular taxa, such as damselflies, but also at certain times of the season when early developmental stages are present. These interactions are further mediated by environmental conditions. For parasites such as *R. ondatrae*, which are most abundant in darkness due to their circadian shedding time, regulation by visual predators may be unlikely. The late developmental stages of the predators and realistic exposure timing may have contributed to the lack of parasite regulation due to consumption observed in the mesocosm experiment. However, this experiment showed that predation on parasites may not be as important in natural systems compared to the trait-mediated indirect effects of predators on hosts. Although we did not detect any behaviorally mediated effects of predators on tadpoles in the laboratory study, this may be due to the larger size and developmental stage of the tadpoles and smaller sizes of the damselflies. In contrast, the tadpoles in the mesocosm experiment did modify their behavior with consequently higher infection levels. Taken together, my results show that it is important to recognize that trophic interactions are not static within communities and can be modified through development, environmental conditions, and trait-mediated effects. Each of these factors may play an important role in how parasite transmission occurs in natural systems.

*Future directions*

The results of my research provide exciting opportunities for continued research that will be widely applicable to host-pathogen systems across a range of aquatic communities. A few
specific areas I have currently been developing include examining transmission at more realistic spatial scales, extending modeling approaches to characterize the functional responses of predators to varying parasites densities, and conducting field investigations. First, it is vital to assess how transmission scales with population size and/or density of hosts and parasites (McCallum et al. 2001). In nature, it is unlikely that all individuals in a population have the opportunity to interact the way that they do in small-scale laboratory studies (McCallum et al. 2001). Consequently, it may be challenging to apply estimated rates or functional forms of transmission from my small-scale environments to more realistic, larger scales, highlighting the need for more relevant empirical tests (McCallum et al. 2001). To extend our knowledge of transmission beyond my dissertation, I conducted a more realistic mesocosm experiment testing two additional spatial scales by constructing 50 L and 150 L mesocosms using the facilities at Hopland Research and Extension Center in Hopland, California (Figure 6.1). I predict that non-linear transmissions apply over the full range of spatial scales tested. To facilitate comparisons among spatial scales, I used Pseudacris regilla tadpoles and R. ondatrae and across a range of host densities (0.04 – 0.16 tadpoles/L) consistent with a previous study with 300 L mesocosms (Johnson et al. 2013). In addition this study design will also allow us to simultaneously examine how spatial scales of transmission influence aggregation of parasites among hosts (Shaw and Dobson 1998, Wilson et al. 2002). Importantly, the 50 L scale matches my previous mesocosms examining the roles of multiple predators allowing me to interpret those results in light of the underlying mechanisms of transmission. Both mesocosm scales more closely represent the small ponds where these species interact in nature. Specimens from this experiment are preserved and awaiting necropsy to assess transmission success, which will then be used in model fitting similar to that used for the laboratory study. This project represents the first large scale test of
Figure 6.1. A. Aquatic mesocosms at Hopland Research and Extension Center, Hopland, CA, used to evaluate the role of spatial scale on transmission. B. Sarah A. Orlofske allocating *Pseudacris regilla* tadpoles to large 150 L mesocosms (left) and small 50 L mesocosms (right).
mathematical models of transmission for a macroparasite and contributes substantially to our understanding of trematode transmission in natural systems.

Another future goal is to combine mathematical models of transmission with models of predation. Although I did not detect a significant reduction in parasite transmission in the presence of predators in my mesocosm experiment, it is important to develop mathematical models to predict how transmission may be altered across a wider range of parasite and predator densities found in nature. An important step towards that goal is to conduct a set of experiments to determine the functional response of the relationship between predator consumption rates and parasite densities, which is needed for model development. Functional responses describe the effect of prey abundance on the predator’s consumption rate and usually have three main types, 1) linear, 2) predation rises linearly then reaches a plateau, or 3) predation rises exponentially at low density then rises more gradually at high prey densities (Murdoch and Oaten 1975). In the future, I plan to conduct a series of experiments to determine how both fish and insect predators respond to changing parasite densities with and without alternative prey. Data from these experiments will be compared to model predictions given each type of functional response and evaluated using maximum likelihood methods. By using a mathematical modeling approach, the results will provide a greater integration of predator ecology and disease ecology, by demonstrating how predator functional responses influence parasite transmission.

Another important knowledge gap is connecting processes identified in the laboratory and patterns of disease observed in natural ecosystems. I propose to fill this knowledge gap through a novel field survey accounting for initial parasite inputs to the system to examine how communities of parasite predators influence transmission and pathology in nature. Significantly, this approach will test hypotheses regarding the relationship between predator community
characteristics and disease transmission in aquatic ecosystems. This fieldwork will address the relative importance of predation in determining patterns of infection and disease including the role of species diversity, abundance or identity in influencing transmission dynamics. This research was part of a larger field study conducted in the San Francisco Bay Area of California, which is an area with one of the highest reported incidences of R. ondatrae induced malformations (Johnson and Sutherland 2003). My approach was to examine a gradient in aquatic predator community diversity coupled with quantifying initial parasite inputs to the system and precisely examining parasite infection and pathology in wild amphibian populations. By using this approach, I can determine the role of predators in mediating infection and pathology, the specific components of predator diversity responsible for this effect, and whether predator communities conform to predictions of community structures supporting the dilution effect. The results of this observational study will be compared to my previous laboratory studies identifying the specific mechanisms, which are more difficult to evaluate given field data alone. Overall, this research will contribute greatly to our understanding of the population level impact of predator communities on parasite transmission and what specific characteristics of predator communities are responsible.

*Implications of ecosystem change for disease dynamics*

My research addresses important applied issues related to biodiversity change, including population extirpation, species extinction, and introduction of non-native species. Anthropogenic environmental changes are associated with the loss of biodiversity world wide leading to altered disease dynamics (Pongsiri et al. 2009). Invasive species are a wide-spread form of environmental change that also contributes to homogenization of communities, which impacts
pathogen transmission (Rahel 2002, Olden et al. 2004, Ostfeld and Keesing 2012). Indeed, Western Mosquitofish (Gambusia affinis) have been introduced to the wetland ecosystems in California on which I based my laboratory studies. The parallel changes in biodiversity and emergence of infectious disease, emphasize the need to understand both the processes simultaneously (Daszak et al. 2000, 2001). My research has shown that it is essential to consider the functional roles of species diversity including both density-mediated effects on parasites and trait-mediated indirect effects on hosts. Based on this framework, future research should be directed at studying both the direct and indirect effects on hosts and parasites due to introduced species in order to predict the consequences for disease dynamics (Pujol-Buxó et al. 2012). Furthermore, understanding which species may play a disproportionate role in mediating transmission and subsequent pathology may provide justification for concentrated conservation effort.

My results suggest that another key to understanding community ecology in general is to better incorporate predator ecology into studies of host-pathogen interactions (Raffel et al. 2008). My transmission modeling has suggested parallels with predator functional responses and parasitoid searching behavior, which links processes previously isolated as distinct fields in ecology (Antonovics et al. 1995, Raffel et al. 2008). Although previously overlooked, studies combining parasites into food webs with free-living species have revealed numerous direct links between predators and parasites (Lafferty et al. 2008, Johnson et al. 2010, Preston et al. 2013). In addition, parasites constitute large amounts of biomass in aquatic ecosystems, which can potentially support predator populations (Kuris et al. 2008, Preston et al. 2013). Traits of parasites, such as high-energy reserves and lack of indigestible exoskeleton, suggest that parasites may be important components of predator diets (Kaplan et al. 2009, Johnson et al
2010). Combined with my research showing that diverse predators consume potentially large numbers of parasites, this indicates that these interactions may be important pathways for biomass and energy flow. Therefore, changes in species diversity may have cascading effects through food web interactions including parasites.
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