Interactions between echinostome parasites and larval anurans across ecological contexts and scales

by

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Abstract

Parasites influence population dynamics and community structure, yet historically have received relatively little attention. Most host-parasite studies focus on these interactions in isolation, despite the likelihood that outcomes depend on the presence of other species (i.e., community context). In this dissertation, I examine the context dependence of trematode parasite (Digenea: Echinostomatidae) interactions with larval frog hosts and the implications for amphibian population dynamics and community structure. First, I address the dependence of host-parasite interactions on predator presence. Like many animals, tadpoles frequently encounter both natural enemies simultaneously, and I show how the presence of echinostomes and odonate predators leads to non-additive effects on important host traits such as physiology, behavior, and development. I further demonstrate that both enemies synergistically affect survival because of such trait effects, i.e., parasite avoidance behavior (higher activity) increases predation rates by enhancing tadpole visibility. These results suggest a potentially general tradeoff between responding to predation risk and parasitism. Second, I address the dependence of host-parasite interactions on the presence of host competitors and resource levels. I show that, at least under some circumstances (i.e., poor growth conditions or high densities), competition exacerbates both infection levels and the negative effects of echinostomes on tadpole growth and survival. I also show that higher resources increase infection levels, due to enhanced tadpole growth rates and size-dependent infection rates. Third, I scale this approach up to a multi-host context, which provides evolutionary insights and suggests important implications for community structure. I show that faster developing and spring-breeding species experience relatively greater infection levels and post-infection mortality, and species with greater variation in habitat use tend to exhibit more plastic behavioral responses to parasites. Finally, I examine parasitism in natural ponds, finding that both biotic and abiotic factors affect echinostome distributions and abundances, and echinostome abundances influence larval frog survival. Overall, my findings emphasize the importance of considering ecological context in understanding and predicting host-parasite interactions, and the consequences to population

dynamics and community structure. Further, since echinostome abundance appears associated with human activities and disease has contributed to recent global amphibian declines, my findings have significant conservation implications.

Chapter I

Introduction

Natural enemies have important effects across ecological scales, from impacts on individual survival and traits to population dynamics (Solomon 1949) to community structure (Holt 1977). Predators historically received the most attention for large scale effects (Paine 1966, Sih et al. 1985), but parasites also play a significant role (Anderson and May 1978, Hudson et al. 1998, Hatcher et al. 2006, Wood et al. 2007). Large-scale effects of parasites are likely common because of strong fitness effects on hosts and a broad distribution within food webs (Lafferty et al. 2006). It is therefore imperative to evaluate parasite effects across scales to develop understanding and make predictions of many species interactions and dynamics.

A critical step in scaling up parasite effects is to assess the context-dependence of host-parasite interactions. Although typically examined as pairwise interactions, host-parasite interactions are inevitably embedded in complex food webs, and the effects of parasites can depend strongly on other species. For example, host-parasite interactions can depend on the presence of hosts' predators (Packer et al. 2003, Johnson et al. 2006, Ramirez and Snyder 2009, Belden and Wojdak 2011) and competitors (Hochberg 1991, Bedhomme et al. 2005). Interactions between parasites and other food web components can thus have unexpected effects on host fitness and parasite transmission.

These interactions may result from trait- or density-mediated effects, although ecologists classically focused only on density effects of natural enemies (Paine 1966, Estes and Palmisan 1974, Carpenter et al. 1985). However, most ecologists now recognize that trait-mediated effects are common (Bolker et al. 2003, Werner and Peacor 2003) and can be of comparable magnitude to density effects (Peacor and Werner 2001), although their application in host-parasite systems is limited. Parasites can mediate such effects because they influence a range of host traits, such as growth (Agnew et al. 2000, Arnott et al. 2000), development (Johnson et al. 2001, Kristan 2002), behavior (Hart 1990, Poulin 1994), and physiology (Thomas et al. 2010, Warne et al.

2010), which can influence interactions with other species. For example, parasites may affect susceptibility to predators (Lafferty and Morris 1996, Hatcher et al. 2006, Duffy and Hall 2008), which can alter population dynamics (Ives and Murray 1997, Fenton and Rands 2006) and community structure (MacNeil et al. 2003). The results of trait effects can thus be strongly context-dependent, and measurements of these effects will be needed to predict the dynamical consequences of parasitism.

In this dissertation, I examine the dependence of host-parasite interactions on ecological context and the consequences at multiple scales (individual host, population, and community), with larvae of common Michigan species of frogs as my study system. Larval amphibians are frequently exploited by parasites and provide an ideal system in which to examine their separate and joint effects with additional biotic stressors. The effects of predators and competition on larval frog traits have been well studied (e.g., Wilbur and Collins 1973, Anholt and Werner 1995, Relyea 2004), but the effects of parasites and interactive effects with other stressors are not yet well understood (but see Thiemann and Wassersug 2000, Johnson et al. 2006, Koprivnikar et al. 2008, Szuroczki and Richardson 2012). Parasite effects on amphibians require special attention, due to recent global declines in which disease is believed to be a major contributing factor (Collins and Storfer 2003, Stuart et al. 2004).

I focus on interactions between larval frogs and echinostome parasites (Digenea: Echinostomatidae). Echinostomes have a complex life cycle involving three hosts: an aquatic snail first intermediate host, a range of potential second intermediate hosts (larval amphibians the hosts of concern here), and a bird or mammal definitive host (Najarian 1953, Kanev et al. 2000). In larval frogs, echinostomes infect the kidneys, which can cause disease and death at an early stage (Schotthoefer et al. 2003, Holland et al. 2007). Echinostomes can also affect important traits, including behavior (Koprivnikar et al. 2006, Rohr et al. 2009) and growth (Fried et al. 1997, Raffel et al. 2010). Echinostomes are widely distributed and common parasites of larval frogs (Skelly et al. 2006, Johnson and Hoverman 2012, Richgels et al. 2013). Effects of echinostomes merit attention from a conservation perspective, as infection is positively associated with anthropogenic activities, such as urbanization (Skelly et al. 2006), agriculture (King et al. 2007, King et al. 2010), and pollution (Rohr et al. 2008). Understanding how parasitism interacts with additional stressors, such as predation and competition, will be important to assess the overall consequences of increased abundances near human activities.

I begin in the next two chapters by examining the separate and joint individual-level effects of parasites and predators. In Chapter II, I focus on nonconsumptive predator effects, which may have dramatic consequences for host-parasite interactions by influencing the ability of prey items to avoid, resist, or tolerate infection (Thiemann and Wassersug 2000, Duffy et al. 2011, Szuroczki and Richardson 2012). Both predators and parasites can affect host traits, such as growth rates (Fried et al. 1997, Relyea 2004) and behavior (Relyea 2001, Rohr et al. 2009), and these effects may in part be mediated through shared physiological pathways (e.g., the glucocorticoid stress hormone, corticosterone [CORT]) (Middlemis Maher et al. 2013). I examine how these natural enemies separately and jointly affect a range of traits in larval frogs, including behavior, physiology, morphology, growth, and development. The results suggest that the combination of parasites and predator presence has both additive and interactive effects on different traits, and the effects of echinostomes are dose-dependent, with potential consequences for species interactions in natural populations.

Chapter III extends these findings to examine interactive effects of echinostomes and predators on larval frog survivorship. In addition to nonconsumptive effects of predators on susceptibility to parasites discussed above, parasites can alter host traits that influence predation risk (Lafferty and Morris 1996, Mouritsen and Poulin 2003). I thus evaluated the combined effects of echinostomes and predators on tadpole survival, and the contribution of different mechanisms to an observed synergistic effect on mortality. The results provide insight into the complex joint effects of natural enemies on hosts and demonstrate a useful general approach to mechanistically understand the interactive effects of multiple natural enemies.

In Chapter IV, I depart from my focus on predator effects to examine the importance of an additional broadly important biotic stressor, competition, on host-parasite interactions. I address two key determinants of the strength of competitive interactions, host density and size structure, which also impact host-parasite interactions. Density may have direct impacts on parasite transmission by altering contact rates between hosts and infective agents (Johnson et al. 2013). In addition, density can have indirect effects, due to increased competition at higher densities that impacts individual susceptibility (Bedhomme et al. 2005, Koprivnikar et al. 2008), potentially including both resistance to and tolerance of infection (Raberg et al. 2009). Both competitive interactions and host parasite interactions can also strongly influence and be influenced by size structure (Morin and Johnson 1988, Fried et al. 1997, Holland et al. 2007),

with potentially important dynamical consequences (Peacor et al. 2007). I address these effects in the tadpole-echinostome system, examining infection and trait effects of parasites on two size classes of larval green frog (*Rana clamitans*) hosts across a density gradient. The results suggest that competition influences both parasite transmission and impacts of parasites on host fitness. Resource levels likely play an important role in these effects, which I examined in a follow-up study discussed in Chapter V. Together, the findings presented in these two chapters suggest that host-parasite interactions depend on host density, competition, resource levels, and size structure, with potential feedbacks between these factors.

In addition to interactions with intraspecific competition and predation, the trait and survival effects of echinostomes likely have important consequences in multi-host species communities. Parasites may influence host community structure (Holt and Pickering 1985, Thomas et al. 1995), and, as discussed above, community context can influence host-parasite interactions. An evaluation of the relationship between biodiversity and disease thus requires measurements of host species' differences in susceptibility to parasites and the dependence of susceptibility on community context (e.g., predator presence). In addition, comparisons among species can provide evolutionary insights into host-parasite interactions and relationships between host traits and susceptibility (Johnson et al. 2012). In Chapter VI, I compare the effects of echinostomes and predator presence on eight species of larval frogs which vary in life history, habitat use, and phenology. The results reveal predictable differences among species in susceptibility dependent on traits, in line with parasite-mediated natural selection, and have implications for community structure and the relationship between biodiversity and disease.

The previous chapters all describe laboratory and mesocosm experimental work, but a clear link to natural distributions and dynamics is necessary. Thus, in Chapter VII, I evaluate the role of several effects and relationships examined experimentally in earlier chapters to patterns in infection and amphibian survivorship in natural ponds. Host traits and a range of abiotic and biotic environmental factors can influence parasite distributions in both snail and amphibian hosts (Hartson et al. 2012, Johnson et al. 2013, Richgels et al. 2013), and variation in amphibian exposure and infection over space and time may influence survivorship. I examined the links between environmental context, parasite distributions, host fitness, and a key population demographic rate (larval survivorship), using a combined field survey and field experiment

approach. The results demonstrate factors likely driving parasite distributions and potential consequences for amphibian population dynamics.

Finally, in the concluding chapter, I integrate the findings of earlier chapters and draw general conclusions on the context-dependence of echinostome-tadpole interactions and the consequences at larger ecological and spatial scales. I then discuss some general implications of these findings for population dynamics, community structure, evolution, and conservation biology. Finally, I outline potentially fruitful directions for future research building upon this work.

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Chapter II

Predators and trematode parasites jointly affect larval anuran functional traits and corticosterone levels

Introduction

Non-consumptive effects of predators can have dramatic consequences across ecological scales (e.g., Creel and Christianson 2008, Peacor and Werner 2001). Visual or chemical predator cues can affect traits such as behavior (e.g., Anholt et al. 2000), growth (Relyea 2004), immunocompetence (e.g., Horak et al. 2006), and development (reviewed in Benard 2004), which can influence interactions with other species. Many of these effects of predators on traits may influence the ability of animals to engage in an effective defense against parasite infection. However, relatively few studies have evaluated the consequences of non-consumptive predator effects for host-parasite interactions (but see Duffy et al. 2011, Ramirez and Snyder 2009, Szuroczki and Richardson 2012, Thiemann and Wassersug 2000 for examples), despite the ubiquity of parasitism within food webs (Lafferty et al. 2006).

The combination of parasites and predator presence may facilitate potential interactions over the entire host-parasite interaction timeline, from the initial detection of parasite presence by the host to infection of the host to host or parasite death. Across this timeline, hosts can engage in three general defense strategies in response to parasites - avoidance, resistance, and tolerance (Medzhitov et al. 2012, Raberg et al. 2009) that may be affected by predator presence. Avoidance refers to behavior that minimizes infection risk, resistance refers to the physiological response by the host to reduce parasite burdens (including immunological resistance to initial infection and later infection clearance), and tolerance refers to the ability of the host to reduce the negative effect of infection on fitness at a set parasite burden. Each of these strategies could be influenced by effects of predators, e.g., on behavior (i.e., avoidance), immune response (i.e., resistance), and body condition (i.e., tolerance), or growth and development rates, which in part

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govern the effectiveness of these strategies. For instance, the ability of hosts to tolerate infection can depend on body size and developmental stage (Holland et al. 2007), which predators can influence (Benard 2004). Such complex effects can have important consequences for host-parasite dynamics (Duffy et al. 2011, Ramirez and Snyder 2009).

Like predators, parasites can influence host traits, including behavior (reviewed in Poulin 1994), development, and growth (e.g., Johnson et al. 2012). However, the strength of these effects relative to those of predators is poorly understood. Additionally, when these natural enemies co-occur, trade-offs and interactions may result, because both parasites and predators can influence the same trait or physiological pathway. In particular, the neuroendocrine stress axis plays an important role in mediating responses of prey to predators (Middlemis Maher et al. 2013) and also to parasites through effects on immunocompetence. The chronic presence of predators can activate this axis (Fraker et al. 2009), which elevates glucocorticoids (i.e., corticosterone [CORT] or cortisol, the primary vertebrate stress hormones, Denver 2009). Host-parasite interactions can also influence and be influenced by this axis, because infectious agents can affect glucocorticoids (Warne et al. 2010), and glucocorticoids can affect the immune response. Acute increases in glucocorticoids can enhance the immune response (Dhabhar 2009), while chronic elevated levels can suppress the immune response (Rollins-Smith 2001) and increase susceptibility to parasites (Belden and Kiesecker 2005).

The separate and combined effects of parasites and predators on survival and traits likely differ among species. Species differentially invest in defense strategies against predators (Cressler et al. 2010) and parasites (Schmid-Hempel 2003) because of variation in costs (e.g., reduced resource allocation to other fitness traits) associated with different factors, such as habitat use (Van Buskirk 2002) or life history (Johnson et al. 2012). A comparison of these differences between species can provide useful insights into potential underlying tradeoffs between susceptibility to different natural enemies, which may have important consequences for community structure.

Here, we focus on the effects of trematode parasites (Digenea: Echinostomatidae) and predators (larval odonates) on larvae of two anuran species, wood frogs (*Rana sylvatica*) and green frogs (*Rana clamitans*), which differ in breeding phenology, habitat use, life history, and other traits. We hypothesized that potentially important interactions may occur due to effects of predator cue on different components of susceptibility through at least five mechanisms. First,

chemical cues from parasites and predators can affect larval frog behavior (Rohr et al. 2009), which may be mediated by CORT (Middlemis Maher et al. 2013), so that the combination may have interactive effects on larval frog CORT and behavior before host-parasite contact occurs. Second, reductions in activity level caused by predator cue (Anholt et al. 2000) may inhibit the parasite avoidance response (which may contribute to a documented predator-induced increase in infection rates, Szuroczki and Richardson 2012, Thiemann and Wassersug 2000). Third, physical contact and subsequent penetration by parasite infective stages in combination with predator cue may interactively affect CORT and traits beyond the effects of parasite cue alone, due to stress associated with tactile cues and short-term (<48 h) physical damage caused by the parasite. Fourth, physiological and behavioral costs associated with prolonged exposure to predator cue may reduce the ability of hosts to eliminate parasite cysts (i.e., a documented form of resistance, Holland 2009). Finally, costs of prolonged exposure to predator cue may also impair host tolerance of infection, resulting in non-additive effects of infection and predator cue on traits (i.e., growth, behavior, development rate, and morphology) and survival. Separately evaluating the effects of predator cue on the response of anurans to parasites across the host-parasite interaction timeline should reveal the extent to which these different interactions occur and the underlying mechanisms. We tested for the above interactions in a series of aquaria experiments.

Methods

Study system

In southeastern Michigan, wood frogs breed in vernal ponds between mid-March and early April and develop to metamorphosis by early July. Green frogs breed from late May until early August in semi-permanent or permanent ponds, and typically overwinter as larvae before metamorphosis. We focus on the effects of *Anax spp.* (Odonata) predators and echinostome parasites, both common natural enemies in Michigan ponds, on larvae of these amphibian species. Echinostomes infect the kidneys of larval amphibians, often causing edema or death in early-stage tadpoles (Holland et al. 2007). Echinostomes have a three-host life-cycle involving a snail first intermediate host, an amphibian, fish, or snail second intermediate host, and a mammal or bird definitive host (Najarian 1953). Free-swimming infective stages, cercariae, exit the snail host and enter the larval amphibian through the cloaca, moving to the kidneys where they encyst as metacercariae.

General notes on animal rearing, predator cue generation, and parasite collection

Egg masses of both amphibians were collected from ponds on the Edwin S. George Reserve (ESGR) in Livingston County, MI, and moved into 300 L pools containing aged well water. After hatching, larvae were fed Purina® Rabbit Chow *ad libitum* until the beginning of experiments. During all experiments, tadpoles were fed 6% of their biomass per day of 3:1 Purina® Rabbit Chow: Tetramin® fish flake mixture. Aged well water was used in experiments unless otherwise noted. Predators were a mixture of late-instar *Anax junius* and *A. longipes* collected from the ESGR experimental ponds. *Anax* were kept in 1 L cups of water with a small piece of screen as a perch, and fed tadpoles ad libitum. To generate predator cue for experiments, we changed the water and fed *Anax* 100 mg of conspecific tadpoles. After feeding, the water from all predators was mixed together to homogenize the cue and divided equally among appropriate aquaria. We added an equal volume of water to non-predator treatments. Because not all predators fed each time, between 15 and 25 predators produced cue on a given day (0.5-0.9 predators per aquarium). To provide a source of parasites, *Helisoma trivolvis* snails were collected from two ponds in Livingston County, MI (hereafter referred to as Sheep Pond [42.539683, -83.94794] and Duck Pond [42.481308, -83.983442]). We determined if snails were shedding cercariae by placing the snails in 60 mL water under a 60 W incandescent light for at least 4 h, which induces the cercariae to leave the snail host. We then examined the cups under a dissecting microscope for the presence of cercariae. To collect and count cercariae for experiments, we followed the protocol outlined by Holland et al. (2007) in which cercariae were counted under a microscope and moved into 60 mL cups of water using a pipette. All cercariae used in experiments were introduced to experimental animals no more than 8 h after leaving the snail host. This research was performed in accordance with University of Michigan UCUCA Protocol #07765.

Parasite identification

Trematode cercariae were identified to family as echinostomes based on morphology after Schell (1985). Infected snails were preserved in 70% ethanol after experiments. Five rediae were dissected from individual *H. trivolvis* from Duck Pond and Sheep Pond, and DNA was extracted using a Qiagen DNeasy Tissue Extraction Kit. The DNA samples were then run through a PCR with digenean-specific primers (Dig12 and LSU 1500R) to amplify a portion of 28s ribosomal DNA. The PCR product was run through gel electrophoresis and purified using a

Qiagen Gel Extraction Kit, and then submitted to the University of Michigan DNA Sequencing Core for sequencing. Chromatograms for each sequence were examined in Sequencher, and clean sequences were then compared to those of known species using NCBI Nucleotide BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Experiments 1 and 2: Effects of parasite cue and predator cue on green frog behavior and CORT

To examine the separate and combined effects of parasite cue and predator cue on larval green frog behavior and CORT (the first mechanism), we performed two aquaria experiments with larval green frogs: Experiment 1 focused on the effects of parasite cue and predator cue on behavior, and Experiment 2 focused on the effects of these cues on CORT levels. In both experiments, we used a 2 x 3 factorial, randomized block design in which tadpoles were exposed to water or predator cue and an empty cage (cage control), a caged uninfected snail (host control), or a caged infected snail (parasite cue treatment, snails from Sheep Pond , ~1 g). Infected snails produced >50 cercariae during initial 4 h screenings, while snails were classified as uninfected if no cercariae were shed during screenings. Cages were constructed from 120 mL plastic cups enclosed in Nitex (~1'x1' piece of Nitex 30 micron mesh, closed with a rubber band), affixed to the side of the aquarium with wire. We used a smaller mesh size than the 75 micron mesh used by Rohr et al (2009), who documented effects of parasite cue on behavior, because we observed that cercariae of the species we employed passed through the larger but not the smaller mesh size during pre-experiment trials. To test the finer mesh, we performed trials in which three caged, infected snails were placed in 500 mL of water in plastic containers underneath a 60 W light. We repeatedly examined the containers over 4 h (after which we turned off the light) and again after 24 h for cercariae presence. We observed >100 cercariae inside cages but none outside the mesh. We are therefore reasonably sure that cercariae were not in direct contact with tadpoles, although we did not dissect any tadpoles from the experiments. We also note that infected snails can vary extensively in the number of cercariae produced both among hosts and over time, which may have exacerbated variance in cue production among tanks, although no bias should have been introduced.

In the behavior experiment (Experiment 1), 10 green frog larvae (Gosner [1960] stage 25, mean mass ± SE = 24.3 ± 0.3 mg, originating from four egg masses collected in the first week of July, 2009) were placed in 8 L (26 x 38 x 14 cm) aquaria on July 31, 2009. We had 10 replicates of each treatment combination. We fed the tadpoles and added predator cue on the mornings of

August 1 and 3 and performed 10 sets of behavioral observations on August 4, during which we slowly approached each aquarium and counted the number of active individuals over a 5 s interval. We also performed three sets of observations of the location of animals relative to the snail cages. Each aquarium was divided lengthwise into four quadrants increasing in distance from the cage, and we counted the number of tadpoles present within each quadrant. The experiment was terminated after five days.

In the CORT experiment (Experiment 2), 20 green frog tadpoles (Gosner stage 25, 27.6 ± 0.5 mg, originating from four egg masses collected during the third week of July) were placed in aquaria on August 12, 2009. More animals per container were used in this experiment than in the behavior experiment in order to have enough tissue for the CORT analysis (~500 mg). We fed the tadpoles and added predator cue once on the first day of the experiment. Forty-eight hours after the addition of predator cue, we rapidly (<1 min) collected all surviving tadpoles with aquarium nets and flash froze them by immersion in a dry ice bath containing 95% ethanol. Tadpoles from each aquarium were collected and frozen together in a single vial, and were used to provide a single aggregated measurement of CORT per replicate. We expected that 48 h would be sufficient to observe the long-term elevated CORT response that tadpoles experience in response to predator cue (Middlemis Maher et al. 2013). We conducted steroid hormone extraction on whole tadpoles as described by Denver (1998) and analyzed whole body CORT content by radioimmunoassay (RIA) as described by Licht and colleagues (1983). Samples from a single study were assayed in a single RIA. Potency estimates from the RIA were corrected for recoveries, and inter- and intra-assay coefficients of variation calculated using a quality control standard averaged 13% and 10%, respectively.

Experiment 3: Effects of parasite exposure and predator cue on wood frog behavior and CORT

To examine the separate and combined effects of predator cue and direct exposure to cercariae on larval frog behavior and CORT, we performed a 2 x 2 factorial aquarium experiment, in which wood frog tadpoles were exposed to water or predator cue and water or echinostome cercariae. This experiment tested for holistic behavioral and physiological responses of tadpoles to the combination of parasites and predator cue, as multiple mechanisms may be involved. Predator cue may inhibit parasite avoidance behavior and thus potentially also increase infection rates (i.e., the second mechanism) and may also impact the behavioral and physiological response to tactile cues and short-term physical damage from parasites (i.e., the

third mechanism). Fifteen wood frog egg masses were collected on April 4, 2010, from Southeast Marsh on the ESGR (42.447101, -83.997627). On May 13, 2010, 10 tadpoles (Gosner stage 26-27, 62.6 ± 2.4 mg) were placed in aquaria containing 8 L water. After a 2 h acclimation period, we added predator cue to appropriate aquaria. After 15 min, we added 250 echinostome cercariae collected from *H. trivolvis* from Duck Pond to the parasite treatment aquaria. Beginning 15 min after addition of cercariae, we performed five sets of observations over 1 h. The following morning (20 h post-exposure), we performed five additional sets of behavioral observations. We then immediately collected and flash froze all animals within aquaria, except for one individual from each aquarium, which was collected and preserved in 70% ethanol for later dissection to assess infection. Whole body CORT content of the combined sample of nine frozen tadpoles was measured as in Experiment 2 (i.e., one measurement per replicate). Because tadpoles here were collected 20 h after predator cue addition, we expected that experimental tadpoles were in transition between short- (4 h) and long-term (96 h) CORT responses to predator cue (Middlemis Maher et al. 2013) at the time of collection. Ethanol-preserved tadpoles were dissected using fine forceps under a dissecting microscope, and we counted the number of echinostome metacercariae in the mesonephri, nephric duct, mesentery and pronephri.

Experiment 4: Effects of predator cue and echinostome infection on wood frog survival and traits

To evaluate the effects of predator presence and echinostome infection on traits and survival, and potential effects of predator presence on cyst elimination, we performed a 2 x 3 factorial aquarium experiment in which wood frog tadpoles were exposed to either water or predator cue and three levels of parasite exposure: 0, 25, and 50 cercariae per tadpole. In contrast to Experiment 3, tadpoles here were exposed to parasites prior to exposure to predator cues, in order to examine post-infection effects of predator cue (i.e., the fourth and fifth mechanisms) removed from potential effects on initial infection establishment (the third mechanism). We set up six replicates on April 27, 2010 (Gosner stage 25, 21.4 ± 0.3 mg, originating from the same egg masses as Experiment 3) and an additional six replicates on May 4 (Gosner stage 25-26, 30.0 ± 0.3 mg). We used relatively small tadpoles because susceptibility to echinostomes decreases later in development (Holland 2009, Holland et al. 2007). Echinostome infection was accomplished by moving individual tadpoles into cups containing the appropriate number of cercariae (from snails from Duck Pond) in 60 mL water and left overnight (12-16 h, to ensure

that the cercariae entered the host). The following morning, experiments were initiated by moving five tadpoles into each 8 L aquaria.

We fed tadpoles and added predator cue to aquaria three times per week throughout the course of the four-week experiment, changed water weekly, and removed dead tadpoles throughout. We performed sets of 10 behavior observations over 2 h on eight days (1, 3, 6, 9, 10, 14, 15, and 21 days post-infection) throughout the four-week experiment. Larvae were weighed after two weeks and again at the end of the experiment. Tadpoles surviving to the end of the experiment were euthanized and preserved in 10% buffered formalin for later morphological analysis, staging, and dissection to assess infection. For morphological analysis, digital photographs were taken of each tadpole from a lateral view. A microscope slide was paced under the tail of each tadpole to keep the body flat and parallel to the camera lens. We analyzed the photographs in ImageJ (Schneider et al. 2012), and measured the body length, body depth, tail length, tail depth, and muscle depth (as per Relyea 2001).

Experiment 5: Effects of predator cue and echinostome infection on green frog survival and traits

We used the same design as Experiment 4 to address the same effects in green frogs, except for the following changes. This experiment used 10 replicates, all initiated on August 6, 2010. Green frog tadpoles (Gosner stage 25, 25.8 ± 0.1 mg) originated from four egg masses collected from the ESGR experimental ponds on July 19, 2010. Behavior observations were performed on nine dates (2, 4, 6, 8, 11, 13, 15, 22, and 28 days post-infection). The laboratory temperatures were higher for the green frog experiment (24-28°C) than the wood frog experiment (19-22°C), and the water used during the green frog experiment was filtered using a reverse osmosis and a UV filter. The filter was installed between experiments because an outbreak of bacterial slime occurred in several aquaria in a separate experiment in late June 2010, which was sourced to the well water in the laboratory.

Statistical analysis

All analyses were performed in the R statistical package v.2.15 (http://www.r-project.org/). We analyzed survival, final mass, behavior, infection, and Gosner stage using mixed models implemented with the lme4, glmmADMB, and ordinal packages, with block included as a random factor. For mass, infection, and developmental stage, individuals were nested within replicates within blocks for analyses. Behavior, including location within aquaria

(proportion of animals in the quadrant furthest from cages) in Experiment 1 and activity (proportion of tadpoles active) in Experiments 1,3,4, and 5, was analyzed using generalized linear mixed models (GLMMs) with a binomial distribution and repeated measures. Infection intensities in Experiment 3 were analyzed using a GLMM with a negative binomial distribution to account for overdispersion. For Experiments 4 and 5, final survival was analyzed using a GLMM with a binomial distribution. For analyses of traits and infection, only measurements from individuals surviving to the end of the experiments were included. Log-transformed final mass was analyzed using a linear mixed model, infection (number of parasites that successfully encysted vs. the number unsuccessful) was analyzed using a GLMM with a binomial distribution, and Gosner stage were analyzed using a cumulative link mixed model (clmm function in ordinal package). Total CORT concentrations in Experiments 2 and 3 were analyzed using ANOVA. Finally, for the morphological analyses, each morphological trait was regressed against body mass to get mass-independent measures of morphology, and then the residuals for each trait were analyzed using MANOVA.

Results

Parasite identification

The closest match for parasites from Duck Pond (used in experiments 1 and 2) was *Echinostoma revolutum* based on 99% sequence similarity in NCBI Nucleotide BLAST (Accession AY222246). The closest match for parasites from Sheep Pond (used in experiments 3-5) was *Echinoparyphium cinctum* based on 99% sequence similarity (Accession AF184260). We note that it is possible that multiple species may have been used in a single experiment, as snails from the same pond may have been infected with different echinostome species. In addition, our ability to identify species using this method is limited by the sequences available in BLAST, and a recent phylogenetic analysis of North American echinostomes suggests that a number of cryptic species are present in the mid-western United States (Detwiler et al. 2010). In particular, *E. cinctum* is thought to be restricted to Europe and lymnaeid snails (Kanev et al. 1998), so our species may be another *Echinoparyphium* species (e.g., *E. flexum*) for which sequences are not available for comparison.

Experiments 1 and 2: Effects of parasite cue and predator cue on green frog behavior and CORT

In the behavior experiment (Experiment 1), the proportion of tadpoles active decreased 84% in the presence of predator cue (Figure 2.1a; $z = -7.95$, $p < 0.001$), and the predator x snail treatment interaction was significant (Likelihood ratio test [LRT], $X^2 = 9.95$, $df = 2$, $p = 0.007$). However, additional analyses revealed that the interaction was not significant if the host control treatment was excluded (i.e., comparing only the infected snail and empty cage, $z = -1.10$, $p = 0.3$). Consequently, our results do not indicate a clear effect of parasite cue on activity level, since the parasite cue treatments did not differ significantly from the cage control. Location of tadpoles in aquaria was not affected by the snail treatment (LRT, $X^2 = 0.23$, $df = 2$, $p = 0.9$) or predator treatment ($X^2 = 0.028$, $df = 1$, $p = 0.9$), and the predator x parasite interaction was not significant ($X^2 = 3.8$, $df = 2$, $p = 0.15$).

In the CORT experiment (Experiment 2), two experimental units were excluded from the analysis because of low recovery of CORT (less than 15%; recoveries ranged from 20-50%) from collected samples. We specified this *a priori* cutoff because low recoveries artificially inflate the estimate of hormone concentration. Whole-body CORT concentrations (pg/mg) of tadpoles were 21% higher in tadpoles exposed to predator cue (Figure 2.1b, $F (1, 43) = 7.59$, $p = 0.009$), but there was no difference between controls and the infected snail treatments ($F (2, 43) = 1.142$, $p = 0.3$), and no evidence for an interaction ($F (2, 43) = 0.49$, $p = 0.6$).

Experiment 3: Effects of parasite exposure and predator cue on wood frog behavior and CORT

Activity levels decreased 16% from day 1 to day 2 ($z = -3.88$, $p < 0.001$), but there was no significant interaction between date and treatment. Activity levels decreased in response to separate exposures to predator cue (Figure 2.2a; $z = -3.34$, $p < 0.001$) and parasites ($z = -4.17$, $p < 0.001$), but their effects were antagonistic in combination (parasite x predator interaction: $z = 3.24$, $p = 0.001$). Infection in tadpoles collected from aquaria exposed to parasites did not differ between animals exposed to predator cue or water ($k = 4.3$, $z = -0.21$, $p = 0.8$; mean infection intensity = 15.9 ± 2.0 metacercariae). Eight experimental units distributed across treatments were excluded from CORT analysis because of low recovery (less than 15%; recoveries ranged from 18-27%), and one more unit was lost because the sample tube broke in the centrifuge. Whole-body CORT concentrations (pg/mg) of tadpoles decreased in response to separate exposures to predator cue and parasites, but the combined effects were antagonistic in combination (parasite x predator interaction: $F (1, 18) = 5.07$, $p = 0.04$).

Experiment 4: Effects of predator cue and echinostome infection on wood frog survival and traits

Relative to controls, wood frog survival decreased 49% and 79% after exposure to 25 and 50 cercariae, respectively (Figure 2.3a; z = -7.17, $p < 0.001$), but predator cue had no effect (z = 0.473, $p = 0.6$) and the interaction was not significant (z = -0.31, $p = 0.8$). Final mass decreased 16% and 21% in treatments exposed to 25 and 50 cercariae, respectively (Figure 2.3b; LRT, $X^2 =$ 17.66, $df = 2$, $p < 0.001$), and decreased 16% after predator cue exposure ($X^2 = 20.45$, $df = 1$, $p < 0.001$). The parasite x predator interaction was not significant ($X^2 = 2.51$, $df = 1$, $p = 0.1$). Activity levels decreased in response to increased parasite exposure (z = -6.61, $p < 0.001$) and predator cue exposure (Figure 2.3c, z = -4.59, $p < 0.001$), and the parasite x predator interaction was not significant (z = 0.43, $p = 0.6$). Parasite infection and predator cue had a negative synergistic effect on final Gosner stage (Figure 2.3d; parasite x predator interaction: z = -2.61, $p = 0.009$). Predator cue affected morphology (approx. $F (1, 44) = 4.04$, $p = 0.005$), but parasite infection had no effect (approx. $F (1, 44) = 1.31$, $p = 0.3$), and the interaction was not significant (approx. $F (1, 44) = 0.69$, $p = 0.6$). Univariate analyses showed that tail depth increased in the presence of predator cue ($F (1, 44) = 4.44$, $p = 0.04$), but the other morphological traits were not significantly affected. The final infection intensities from the 25 and 50 cercariae treatments were mean ± SE = 18.6 ± 0.6 and 29.2±1.9 metacercariae, respectively. The number of metacercariae encysted was lower in tadpoles infected on the second start date (z = -2.96, $p = 0.003$) but did not differ between predator treatments (z = -0.24, $p = 0.8$), and a lower proportion of cercariae were encysted in the 50 than 25 cercariae treatment (z = -3.93, $p < 0.001$).

Experiment 5: Effects of predator cue and echinostome infection on green frog survival and traits

Relative to controls, green frog survival decreased 15% and 35% after exposure to 25 and 50 cercariae, respectively (Figure 2.4a; z = 03.07, $p = 0.002$), but there was no effect of predator cue (z = 0.93, $p = 0.35$) or evidence for an interaction (z = -1.23, $p = 0.2$). Final mass decreased 13% and 17% in treatments exposed to 25 and 50 cercariae, respectively (Figure 2.4b; $X^2 = 9.93$, $df = 1$, $p = 0.002$), but the predator effect and the parasite x predator interaction were not significant ($p > 0.9$). Activity level decreased with parasite exposure level (Figure 2.4c; z = -2.94, $p = 0.003$) and in the presence of predator cue (z = -4.20, $p < 0.001$), but the parasite x predator interaction was not significant (z = 0.47, $p = 0.64$). Gosner stage was not significantly affected by parasite exposure level or predator cue, and the predator x parasite interaction was not significant (all effects: $p > 0.4$). Predator cue significantly affected morphology (approx. *F*

(1, 42) = 4.19, p = 0.004), parasite infection had no effect (F (1, 42) = 1.76, p = 0.14), but the parasite x predator interaction was significant (approx. F (1, 42) = 2.59, p = 0.042). Univariate analyses indicated that body depth increased (F (1, 43) = 6.08, p = 0.02) and body length decreased (F (1, 43) = 4.25, p = 0.045) in response to predator cue, and body depth decreased at higher parasite exposure levels (F (1, 42) = 6.74, p = 0.013), but other morphological traits were not significantly affected by parasite infection, predator cue, or the interaction. The opposite effects of parasite infection and predator cue on body depth suggest that the effects of predator cue and parasites were antagonistic. The final mean infection intensities from the 25 and 50 cercariae treatments were 18.5 ± 1.0 and 31.6 ±2.1 metacercariae, respectively. The proportion of metacercariae encysted did not differ among predator treatments or between parasite exposure levels (all effects: p > 0.6).

Discussion

Our study addressed the effects of predator cue on multiple components of parasite susceptibility across the host-parasite interaction timeline, including avoidance behavior, resistance to initial infection, clearance and tolerance. In particular, we examined the effects of parasite cue and predator cue on green frog behavior and CORT (Experiments 1-2), the effects of simultaneous exposure to cercariae and predator cue on wood frog behavior and CORT (Experiment 3), and the post-initial infection effects of predator cue on survival, traits, and clearance in both species (Experiments 4 and 5).

Echinostomes and predator cue strongly affected traits of wood frogs and green frogs, and echinostome infection decreased survival of both species, although several effects were stronger or only significant for wood frog larvae. Notably, the effects of echinostome infection on survival and traits were dose-dependent and caused changes similar in magnitude to the effects of predator cue for both species. Given that the infection intensities here are well within the range of those observed in natural populations (Skelly et al. 2006) and non-consumptive effects of predators can be of comparable magnitude to consumptive effects (Peacor and Werner 2001), the observed effects of parasites likely have important ecological consequences.

Of the hypothesized interactive effects of parasites and predator cue, we found evidence for novel interactive effects of echinostomes and predators on CORT and traits (behavior and morphology), while other effects were additive. Dissection results suggest that this interaction

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was likely not due to differential infection rates in containers exposed to predator cues. Rather, these interactive effects suggest that other tradeoffs may occur between responding to each natural enemy, potentially because tadpoles are constrained in their ability to respond physiologically and behaviorally to a combination of natural enemies. Host investment in immune response may inhibit an individual's ability to invest resources in responding to other stressors (Schmid-Hempel 2003), such as predators. This potential tradeoff between immune response and predator response may be mediated in part by the CORT response. Such a tradeoff may be common in other systems, given the general influence of glucocorticoid hormones on physiology and behavior (Denver 2009).

The timing of the CORT response is important to consider in interpreting our results. Predator cue generally causes a short term (within 4 h) decline in CORT levels that mediate rapid behavioral defenses (i.e., reduced activity levels, Fraker et al. 2009), followed by longer-term (96 h) elevated CORT levels that mediate morphological defenses (Middlemis Maher et al. 2013). The elevated CORT levels in response to predator cue observed in the green frog CORT experiment after 48 h are consistent with longer-term elevated levels. In contrast, in the wood frog experiment, the CORT levels were likely in transition between the short and longer-term responses at 20 h post predator cue exposure. A possible explanation for the observed interaction is that the CORT response is adaptively prioritized to improve survival depending on context. In the absence of predators, a reduction in CORT in response to parasites could allow for elevated immune function, because CORT can be immunosuppressive (Apanius 1998). However, the combination of natural enemies requires a faster morphological change than predators alone, since inactivity (the behavioral defense against predators) is counter-productive against parasites (Daly and Johnson 2011, Koprivnikar et al. 2006, Szuroczki and Richardson 2012), and the presence of cercariae can amplify risk from predators (Belden and Wojdak 2011, Marino and Werner, in press). Therefore, the transition from the short- to the longer-term CORT response to predators (Middlemis Maher et al. 2013) may be accelerated in the presence of parasites in order to induce more rapid morphological change. Future experiments varying the timing of CORT measurements relative to parasite and predator cue exposure should further elucidate this interaction.

A physiological interaction may also contribute to the observed interactive effects of echinostome infection and predator cue on green frog morphology (Experiment 5) and wood frog

development rate (Experiment 4). With respect to morphology, the potential consequences of the interaction for defenses against predators (e.g., Relyea 2001) are unclear, but the opposing effects of predator cue and parasite infection on body depth are consistent with a potential tradeoff. The effects of predator cue on tadpole morphology are well-documented as an inducible defense (e.g., Relyea 2001, Van Buskirk and McCollum 2000), so that a counteractive effect of parasite infection on morphology may be costly.

However, we did not find evidence for the other hypothesized interactions between predator cue and echinostomes. Surprisingly, we did not find any effects of parasite cue and no evidence for increased activity in response to cercariae presence, although these effects have been documented elsewhere (e.g., Rohr et al. 2009, Szuroczki and Richardson 2012). We also found no evidence for an effect of predator cue on initial infection based on the subset of tadpoles examined in Experiment 3, in contrast to other studies (Szuroczki and Richardson 2012, Thiemann and Wassersug 2000). Differences between studies (focal species, experimental venue, timing and length of exposure to cue, echinostome species, and Nitex mesh sizes) may contribute to this disparity. We also found no evidence for the hypothesized effects of predatory stress on cyst elimination (i.e., clearance) in Experiments 4 and 5. After controlled exposure to parasites in the absence of predators, predator cue administered for four weeks afterwards did not influence final infection intensities in either species. Finally, we did not find any evidence for interactive effects of infection and predator cue on growth or survival, suggesting that the observed physiological and behavioral interactions may have limited direct fitness costs, although longer-term or indirect fitness costs (e.g., increased predation susceptibility or reduced competitive ability) may also occur. This result contrasts with that of Koprivnikar (2010), who reported an interactive effect of echinostomes and predator cue on larval leopard frog survival (apparently partly driven by an unexplained positive effect of echinostomes on survival in the absence of predators, which did not occur for either species here). The generally additive nature of many effects of parasite infection and predator cue on traits and survival observed in the predator cue-infection experiments (Experiments 4 and 5) was unexpected, given the complex relationships between predation risk and trait expression (e.g., Relyea 2001) and between host physiology and parasitism (Blaustein et al. 2012).

Given the similar design of Experiments 4 and 5, a general comparison between wood frogs and green frogs may provide useful insights, with important caveats given the above noted

differences between experiments. The apparent greater susceptibility of the faster-developing wood frogs compared to the slower-developing green frogs is in line with a tradeoff between developmental rate and parasite susceptibility in larval amphibians (Johnson et al. 2012), consistent with a more general tradeoff between growth and mortality rates (Schiesari et al. 2006). However, other differences between these species (e.g., breeding phenology, habitat use) besides development rate also have influenced historical and current echinostome exposure and infection rates of these species and likely contribute to the differential effects of echinostomes. Wood frogs breed earlier in the season than green frogs, which decreases their exposure to echinostomes in natural ponds, as echinostome prevalence tends to peak seasonally in mid-summer (Raffel et al. 2011). Additionally, these species differ in their breeding habitat preferences. Green frogs breed in more permanent ponds than wood frogs, a pond characteristic which influences distributions of snail hosts (Hoverman et al. 2011) and thus exposure to echinostomes. If echinostomes are an important selective agent in these ponds, species which have stronger associations with echinostomes could exhibit lower susceptibility to infection. These differences in exposure levels may partly explain why green frogs experience lower mortality and fewer trait effects associated with echinostome infection than wood frogs. A comparison of more species under controlled circumstances would provide additional insights into the underlying mechanisms behind differential susceptibility across species.

Our results offer a useful contrast to those of Thiemann and Wassersug (2000), who examined the effects of echinostomes and banded killifish (*Fundulus diaphanous*) predator cues on green frogs and wood frogs, but did not observe many of the effects of echinostomes observed here (i.e., reduced survival and growth in both species and an interactive effect with predator cue on wood frog development and green frog morphology). A likely contributing factor to this difference is that we used earlier stage tadpoles than in their study (consistent with stage-dependent differences in susceptibility, Holland et al. 2007). However, in agreement with their results, we observed decreased activity in response to echinostome infection in both species. Thiemann and Wassersug explained this decrease by invoking an adaptive response of tadpoles to decrease contact with parasites, which makes sense in the context of their study in which tadpoles were repeatedly exposed to parasites over the duration of their experiments (7 days for wood frogs and 28 days for green frogs), and may potentially contribute to the observed behavioral interaction here in Experiment 3. However, this explanation seems unlikely to explain

our results in Experiments 4 and 5, because the effects of parasites on behavior continued weeks after exposure to cercariae, and tadpoles can have fine-tuned behavioral responses to natural enemies (Fraker 2008). An alternative hypothesis is that lowered activity is a consequence of the physiological costs of infection, as strong physiological effects of infection have been documented at similar infection intensities (e.g., edema, Holland et al. 2007). This reduction in activity likely contributes to the decrease in growth rates of both species in response to parasite exposure. This behavioral response to infection is distinct from the behavioral avoidance response to cercariae (Koprivnikar et al. 2006), which results in increased activity levels. Notably, these two responses may be in opposition, which may have important consequences for parasite transmission and the host's physiological response to infection.

Our results also provide useful insights into effects of echinostomes for which the evidence from the literature has been equivocal, although our findings also create some further ambiguities. For instance, consistent with our findings, echinostomes have been documented to have negative effects on growth and development in some studies (e.g., Orlofske et al. 2013), while others found no effect on growth (e.g., Holland et al. 2007, Raffel et al. 2010). Such differences likely occurred because host-parasite interactions, like predator-prey interactions (Relyea 2001, Relyea 2004), are species- and context-dependent. The outcome of tadpole-echinostome interactions can depend on tadpole size (Holland 2009, Holland et al. 2007), species (Holland 2010, Rohr et al. 2010), and dose, and potentially also echinostome species, parasite exposure duration, and the number of exposures to cercariae.

Both the additive and interactive effects of predators and echinostomes observed here have potentially important ecological and conservation implications. The large effects of predators and parasites on survival, growth, and behavior could dramatically impact other interactions within food webs and alter the consequences of parasitism for population dynamics (Anderson and May 1978). Interactions between the effects of predators and parasites provide additional challenges to measuring interspecific interaction strengths, which will be essential in developing predictive models of natural enemy ecology. From a conservation perspective, echinostome-amphibian interactions are of particular concern, because echinostome infection is higher in ponds in urbanized or agricultural areas (King et al. 2010, Skelly et al. 2006). Ecologists will need to better understand the overall effects of echinostomes to predict the consequences of human landscape modifications for amphibian communities. More generally,

understanding the consequences of combined stressors for amphibians, and whether their effects are additive or interactive, will be an important step as we try to understand the factors driving global amphibian declines, including disease (Blaustein et al. 2012).

Acknowledgements

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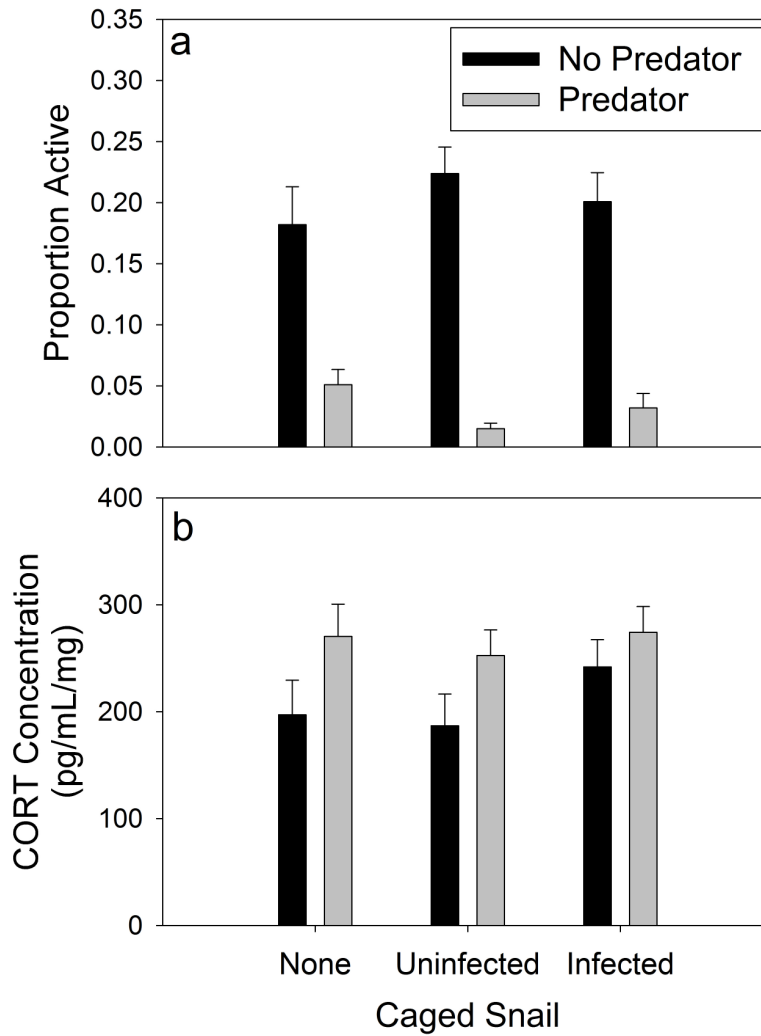
Figure 2.1: a) Predator cue reduced the proportion of green frog tadpoles that were active in Experiment 1 (p < 0.001) and b) raised whole-body CORT concentration of tadpoles in Experiment 2 relative to controls ($p = 0.009$). Tadpoles were exposed to water (black bars) or predator cue (gray bars) and empty cages, caged uninfected snails (host control), or caged infected snails (parasite cue treatment). Bars represent mean ± s.e.m.
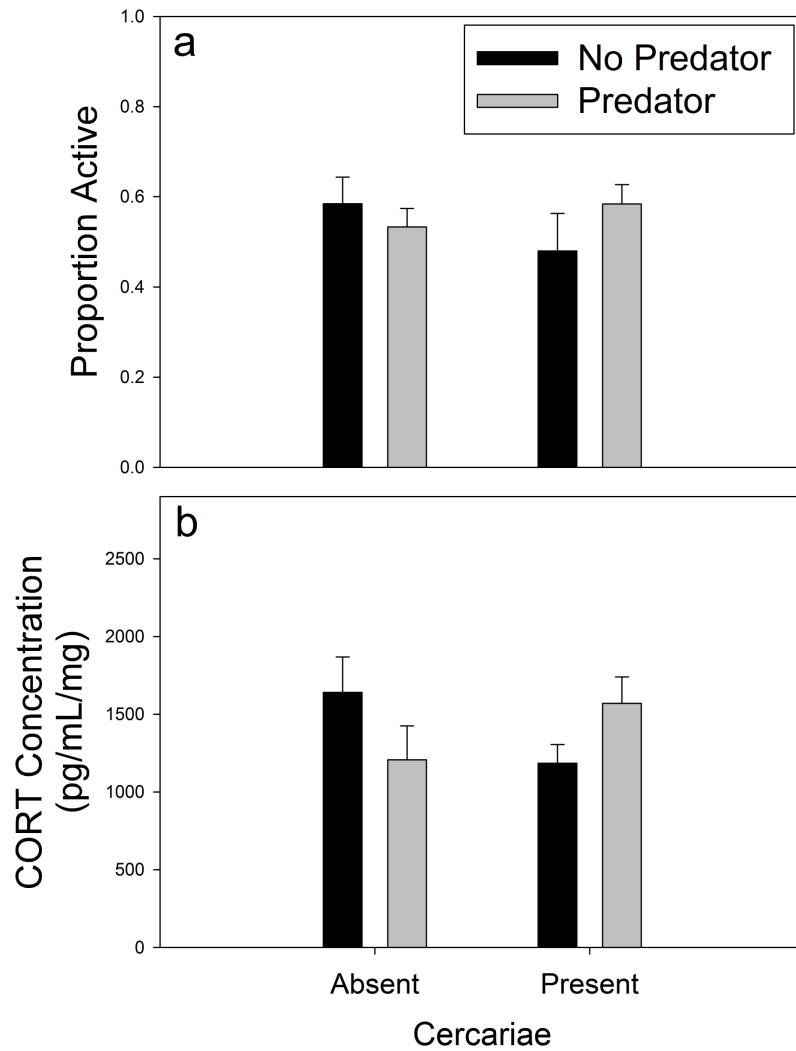
Figure 2.2: Predator cue and the presence of cercariae had interactive effect on a) activity levels (p = 0.001) and b) whole-body CORT concentration (*p* = 0.04) of wood frog larvae in Experiment 3. Larvae were exposed to 0 or 250 echinostome cercariae and water (black bars) or predator cues (gray bars). Bars represent mean ± s.e.m.
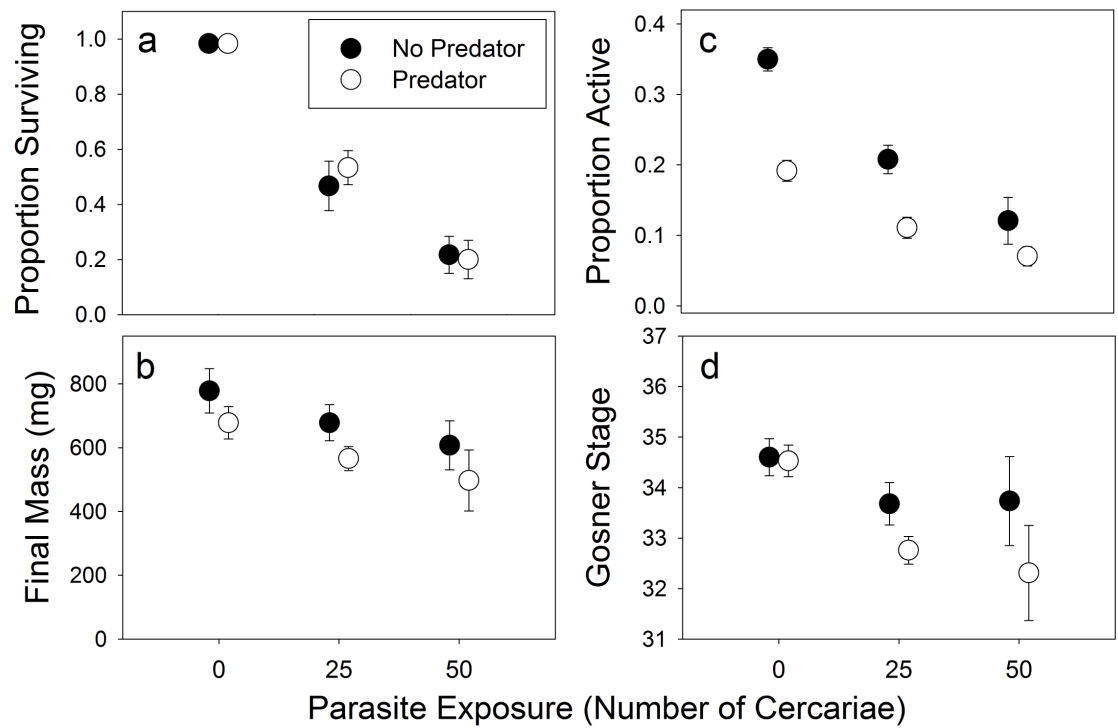
Figure 2.3: Mean ± SE a) final survival (number of individuals), b) final mass, c) proportion of tadpoles active, and d) Gosner (1960) stage of wood frog tadpoles exposed to three levels of echinostome cercariae (0, 25, or 50) and water (solid circles) or predator cues (empty circles) in Experiment 4. Points are offset to show error bars.
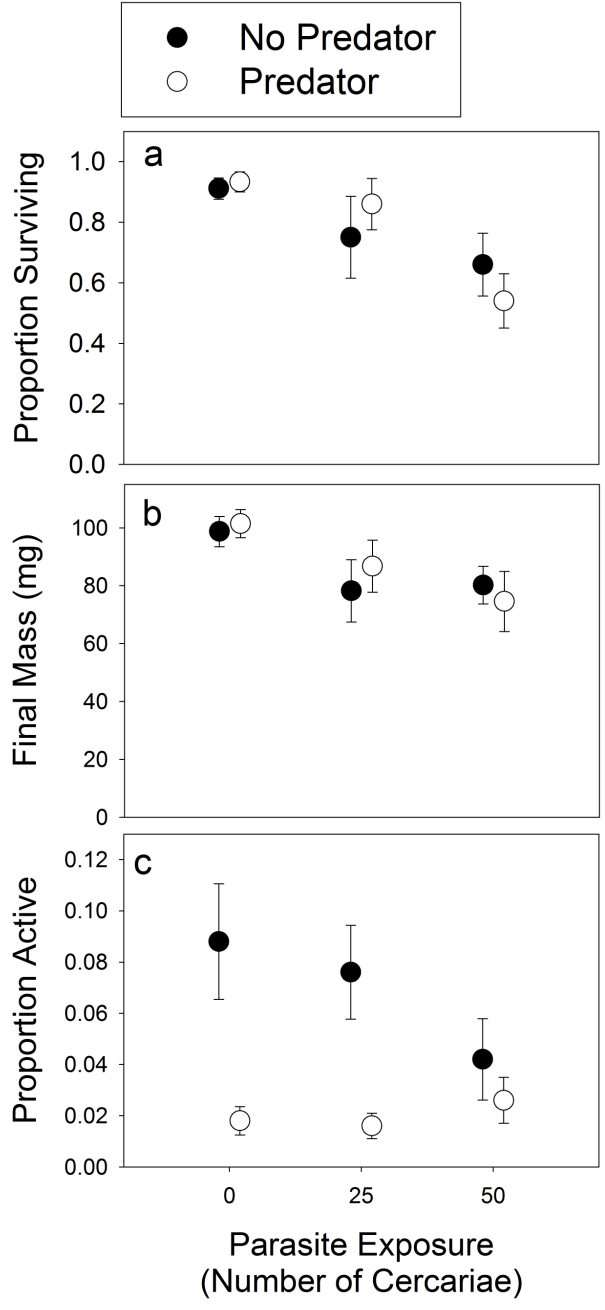
Figure 2.4: Mean ± SE a) final survival (number of individuals), b) final mass, and c) proportion of tadpoles active for green frog tadpoles exposed to three levels of echinostome cercariae (0, 25, or 50) and water (solid circles) or predator cues (empty circles) in Experiment 5. Points are offset to show error bars.

Chapter III

Synergistic effects of predators and trematode parasites on larval green frog (*Rana clamitans*) survival

Introduction

Species are inevitably embedded in complex food webs, in which they interact with multiple natural enemies, competitors, and resources. Classically, interactions between these species have been studied pairwise in isolation, despite the acknowledged importance of higher order interactions (Wootton 1994, Peacor and Werner 2004). In particular, trait-mediated effects are a widespread source of higher order effects and can be comparable in magnitude to density-mediated effects (Bolker et al. 2003, Werner and Peacor 2003). A common source of trait-mediated effects are interactions between parasites and predators (reviewed in Hatcher et al. 2006), which can have non-additive, often synergistic, effects on a shared group of victims (e.g., Johnson et al. 2006, Ramirez and Snyder 2009, Duffy et al. 2011). These non-additive effects are understudied, despite evidence that there may be important implications for population dynamics (e.g., Hudson et al. 1992, Ives and Murray 1997, Dwyer et al. 2004, Fenton and Rands 2006) and community structure (e.g., Thomas et al. 1998, Lefevre et al. 2009). Understanding both the relative strength of such effects and the underlying mechanisms will be crucial to developing a predictive theory of natural enemy ecology.

Predators and parasites in combination may have interactive effects on shared victims through several mechanisms. For instance, parasites may affect host traits, such as behavior (Poulin 1994, Rohr et al. 2009) and growth (Palacios et al. 2012), which can influence susceptibility to predators (e.g., Kagan 1951, Lafferty and Morris 1996, Behringer and Butler 2010). Such host trait modifications may reduce costs of parasitism for hosts (e.g., anti-parasite behavior, Hart 1990) or increase parasite fitness (e.g., parasite-increased trophic transmission, Lafferty 1999, Lagrue et al. 2007), although many trait effects of parasites are not necessarily

adaptive (Poulin 1995). These effects on traits may affect predation rates by increasing predator-prey encounter rates or reducing prey escape ability. In addition, nonlinearities in predator-prey (e.g., Type II functional response, Holling 1959) or parasite-host interactions (e.g., Diaz and Alonso 2003, Luong et al. 2011) may lead to non-additive effects. For example, predators can reduce host densities, which may increase the ratio of parasite infective stages to hosts, thereby resulting in higher per capita infection rates. These density-mediated effects of predators on infection rates may lead to an interactive effect on mortality, if mortality increases nonlinearly with infection intensity. Finally, the presence of predators may influence traits of prey, such as behavior (Relyea 2001a), growth (Relyea 2004), or immunocompetence (Horak et al. 2006), that in turn influence susceptibility to parasites (Ramirez and Snyder 2009, Duffy et al. 2011). Such trait changes may be adaptive prey defenses (Van Buskirk and McCollum 2000, Relyea 2001b) or non-adaptive byproducts of other trait changes (Bourdeau and Johansson 2012). The consequence of these predator-induced trait changes may be higher infection rates or reduced tolerance of infection. All of these mechanisms could potentially drive interactive effects that may have important implications for parasite transmission and population dynamics.

Here, we examined the separate and combined effects of predators and trematode (Digenea: Echinostomatidae) parasites on larval frogs, and then we evaluated potential underlying mechanisms that were responsible for interactions. Larval frogs exhibit an array of trait effects in response to parasites (e.g., Rohr et al. 2009, Raffel et al. 2010) and predators (e.g., Relyea 2001a, 2004), which can drive interactive effects (Thiemann and Wassersug 2000, Belden and Wojdak 2011). First, we hypothesized that parasites (echinostomes) and predator (larval odonate) cues interact synergistically to decrease tadpole survival, because of a documented positive effect of visual and chemical predator cues on echinostome infection intensity (Thiemann and Wassersug 2000, Szuroczki and Richardson 2012). Second, we hypothesized that echinostomes further interact with direct predation to decrease survival, because parasites can affect traits involved in predation susceptibility. To test these hypotheses, we conducted a large-scale mesocosm experiment in which we examined the effects of echinostome parasites in combination with caged predators (i.e., non-consumptive effects) and direct predation (i.e., consumptive and non-consumptive effects) on traits and survival. As a key next step, we then performed a series of follow-up experiments to evaluate the contribution of potential mechanisms driving synergistic effects.

Methods

Study system

Echinostomes infect the kidneys of larval frogs, causing edema and often death at high infection intensities in early Gosner (1960) stage tadpoles (Holland et al. 2007). In addition, echinostomes can also affect larval amphibian growth (Fried et al. 1997) and behavior (e.g., parasite avoidance, Koprivnikar et al. 2006, Rohr et al. 2009). Echinostomes have a complex life cycle involving three hosts and can exploit multiple host species during each life stage (Kanev et al. 2000). A free-living miracidium hatches from an egg released in the feces of the definitive host (mammal or bird), which infects a snail host. Within the snail host, the parasite undergoes asexual reproduction during sporocyst and multiple redia stages before a free-swimming cercaria stage is released. Cercariae then infect the second intermediate host (snail, larval amphibian, or fish). In larval amphibians, cercariae enter via the cloaca and encyst in the kidney, forming metacercariae. When the definitive host consumes the second intermediate host, the parasite develops into an adult in the definitive host's digestive tract and reproduces sexually.

Green frog (*Rana clamitans*) larvae are common hosts for echinostomes in ponds in the eastern and central United States (Najarian 1954, Skelly et al. 2006). At our study site in southeastern Michigan, green frogs breed from late May to early August, and larvae typically overwinter in ponds before metamorphosis.

General Methods and Animal Care

Tadpoles used in experiments were from egg masses collected from the Edwin S. George Reserve (ESGR) experimental ponds and moved into 300 L pools containing aged well water. After hatching, tadpoles were fed Purina® Rabbit Chow ad libitum until the beginning of experiments. Gosner (1960) stage 25 tadpoles were used at the initiation of experiments in both mesocosms and aquaria. Aquaria experiments occurred in plastic boxes (26 x 38 x 14 cm) filled with 8 L of water, during which tadpoles were fed 6% of their biomass per day with 3:1 Purina® Rabbit Chow: TetraMin® Fish Flake mixture every 2-3 days. Water used in the laboratory was reverse osmosis and UV filtered well water with 63 mg/L of API aquarium salt added.

Predators were a combination of late-stage larval odonates *Anax longipes* and *A. junius* (common predators of larval frogs) collected from the ESGR experimental ponds. Parasites were from *Planorbella trivolvis* snails, a first intermediate host of echinostomes, collected from three

natural ponds on the ESGR and from two nearby ponds, Duck Pond (42.481308, -83.983442) and Kaiser South Pond (42.430299, -84.036582), in Livingston County, MI. To determine infection status, snails were placed in 60 mL water 10 cm underneath a 60 W lamp for at least 4 h to stimulate cercariae shedding. Cercariae were identified to family (i.e., Echinostomatidae) using a taxonomic key (Schell 1985). This research was performed in accordance with University of Michigan UCUCA Protocol #07765.

Experiment 1: Combined effects of echinostomes and predators

We performed a 3 x 2 factorial experiment in mesocosms (1,300 L cattle watering tanks; 150 cm diameter x 75 cm depth) to examine the effects of predators, echinostomes, and their combination on larval green frogs. The three predator treatments consisted of no predators, two individually caged *Anax*, and two free *Anax*. Caged *Anax* release chemical cues and thus allow us to examine the nonconsumptive effects of predators independent of consumptive effects. Each caged predator was fed 300 mg green frog tadpoles three times per week throughout the duration of the experiment. The cages were constructed from a 10 x 10 cm piece of slotted drain pipe with the ends covered with window screen attached by rubber bands. Each cage contained a small piece of polystyrene so that it would float at the surface of the mesocosm. Empty cages were placed in containers in the other treatments. We manipulated the presence of parasites in tanks by stocking either three infected or uninfected *P. trivolvis* snails (~1g). The three snails were placed together into a cage (same type as for predators) along with three pieces of polystyrene, and the cage was placed into the appropriate treatment. Each treatment combination was replicated five times, and we used a randomized block design.

Between June 30 and July 1, 2010, the cattle tanks were filled with well water and covered with 60% shade cloth to exclude colonization by other frogs and predators. On July 2, we inoculated each tank with zooplankton and phytoplankton, and we added 300 g of leaf litter (mostly *Quercus*) to provide a natural substrate and 25 mg Purina® Rabbit Chow to provide food and nutrients. On July 5 and 6 (day 1 and 2), we added 200 green frog larvae (mean ± SE = 12.7 ± 0.6 mg, originating from six egg masses [collected on June 17 and 18]) to each tank. Caged snails and predators were then added to appropriate containers on the evening of day 2. We also placed a cage in each mesocosm containing 10 green frog tadpoles on day 5. Infection of caged tadpoles provided a separate measure independent of any selective predation by free predators that may have affected the observed infection in non-caged tadpoles. Cages (30 x 45 x 5 cm)

were constructed of window screening covering a frame of plastic fencing. Tadpoles were added to each cage along with three pieces of polystyrene, and the cage was closed with two plastic zip ties. The cages were removed on day 24, and surviving tadpoles were counted, weighed, euthanized, and preserved in 70% ethanol for later dissection.

To measure behavior, two observers conducted observations of tadpoles by slowly circling each tank and counting the number of visible individuals that were active (moving) using scan sampling (Altmann 1974). We performed five replicate sets of observations over 2 h on four dates (days 8, 12, 18, and 23), all occurring between 0800 and 1800. To estimate infection midway through the experiment, 10 tadpoles from each tank were removed, euthanized, and preserved in 70% ethanol on day 14. To estimate survival midway through the experiment, we took a standardized sample of tadpoles from each tank on day 17. First, we used a "pipe sampler" to sample all animals within a 0.1 m^2 of water column within each tank. The pipe sampler was constructed of 76 cm length of 36 cm diameter aluminum pipe fitted with handles at the top. The sampler was quickly thrust down in the center of each tank to trap any animals within the column, and all animals within were counted by sweeping through with a dip net (22×27 cm with a 1× 2 mm mesh size) until we had 10 sweeps that captured no animals. Second, we performed an additional 10 sweeps of the dip net through other regions of the tank and counted the number of individuals captured with each sweep. The total number of tadpoles captured was used to estimate survival, and all animals were returned to the tank at the end of sampling.

The experiment was terminated on days 26 and 27. We haphazardly selected and weighed 25 individuals (or all, if fewer survived), and then all tadpoles were euthanized and preserved in 10% buffered formalin. To measure infection intensity, five tadpoles collected on day 14 and five from the end of the experiment (except one container where only one individual survived) were dissected under a microscope using fine forceps. We also dissected five tadpoles (or all surviving when fewer) from the mesh cages in each tank. Unfortunately, we could not measure infection intensity of tadpoles that died during this and subsequent mesocosm experiments because they were consumed by predators or decomposed rapidly before the end of experiments. During dissections, we counted the number of metacercariae present in the mesonephri, nephric ducts, and pronephri.

Survival was analyzed using ANOVA to make three orthogonal comparisons: no predator vs. caged predator, no predator vs. free predator, and caged predator vs. free predator. These comparisons allowed us to assess the contribution of nonlethal predator effects to the overall effects of predators separately and in combination with parasites. We analyzed both mid-experiment (day 17) estimated survival and final (day 26) survival, because an interaction may be difficult to detect if few individuals in some treatments survived to the end of the experiment. Final mass and Gosner stage were analyzed using MANOVA. Survival, mass, and stage were log-transformed prior to analysis, because a multiplicative model better represents the potential interactive effects of multiple natural enemies (Vonesh and Osenberg 2003). Activity (mean proportion active across dates) was analyzed using ANOVA only for the no predator vs. caged predator comparison, because few or no tadpoles were visible in the free predator treatment containers on most dates to calculate activity. Activity was arcsine-square root transformed to improve normality. The mean number of encysted metacercariae (day 14 and final) across predator treatments was analyzed using repeated measures ANOVA. Log-transformed survival, final mass, and infection of caged tadpoles were analyzed using ANOVA. All analyses in this study were performed in the R statistical package v.2.15 (http://www.r-project.org/).

Follow-up experiments to evaluate potential mechanisms

Following Experiment 1, we performed a series of additional experiments to examine four potential mechanisms underlying interactive effects of predators and parasites. These mechanisms include: A) Cercariae may affect traits (e.g., parasite avoidance behavior, Koprivnikar et al. 2006, Rohr et al. 2009) that cause increased visibility and higher predator encounter rates, thereby leading to increased predation susceptibility. B) Infection may affect traits that impair predator escape ability, thereby leading to increased predation susceptibility. C) Decreases in density due to consumption by predators may increase per-capita infection rates, if infection rates are density-dependent. D) Predator cue may increase infection rates or reduce tolerance through effects on host behavior and physiology.

Experiment 2: *Effects of cercariae exposure on predation risk*

To measure the effects of cercariae exposure on predation susceptibility (Mechanism A), we performed a set of predator trials in aquaria in which tadpoles were exposed to *Anax* in the presence or absence of cercariae. On August 26, 2011, 10 tadpoles (42.6 ± 2.3 mg, originating from eight egg masses [collected on July 15 and 26]) were placed into forty aquaria. After 30

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minutes, we added one infected or uninfected snail to each aquarium. Thirty minutes thereafter , we added a small amount of predator cue (water from five 1 L containers containing *Anax* fed 100 mg tadpoles was divided among aquaria, ~100 mL cue per aquarium) to alert tadpoles of impending predator presence and finally placed one *Anax* into each aquarium. *Anax* were sorted visually by size, and comparably sized *Anax* were used in the uninfected and infected snail treatments. Aquaria also contained a piece of window screen (~3 x 30 cm) to provide a perching structure for *Anax*. We counted the number of surviving tadpoles in each container every 30 min and terminated the experiment after 6 h. We compared time to the first predation event in each aquarium using Cox proportional hazards survival analysis (the coxph function in the R survival package).

Experiment 3: Effects of echinostome infection on predation risk

To evaluate the effects of echinostome infection on predation susceptibility (Mechanism B), we performed a series of predator trials in mesocosms. Groups of tadpoles were exposed to three infected or uninfected snails and empty cages or two caged predators in an initial set of mesocosms (hereafter, exposure tanks), and then subsets of tadpoles were moved to a new set of cattle tanks (hereafter, trial tanks) where predator trials were performed in the absence of cercariae (i.e., post-exposure). Exposure tanks were thus set up using a 2 x 2 factorial design similar to the caged vs no predator treatments in Experiment 1. These treatments allowed us to assess the effects of echinostome infection and prior exposure to predator cue on predation rates during predator trials. The presence or absence of caged predators was manipulated to assess whether parasitism inhibits adaptive trait-responses to predators, which anuran larvae can exhibit (Relyea 2001b). Exposure tanks were 32 1,300 L cattle tanks set up as in Experiment 1 (tanks filled and leaf litter added June 20-21, 2011, inoculated with plankton and rabbit chow added on June 24). We added 250 tadpoles (16.0 ± 0.6 mg, originating from seven egg masses [collected on June 8 and 10]) to exposure tanks on June 27 and caged predators and snails on June 29.

Two sets of predator trials were conducted in 32 trial tanks 8 and 15 days after treatments were instituted in exposure tanks. Trial tanks were filled with well water and covered with 60% shade cloth on July 1, and 300 mg leaf litter was added before each trial. One and two weeks after the predator and parasite treatments were instituted in the exposure tanks, we haphazardly moved 40 individuals from each exposure tank into a trial tank, thereby removing them from exposure to cercariae and predator cues. Additional samples were also collected from exposure

tanks on each date to estimate mass (10 tadpoles on each date) and infection intensity (5 tadpoles dissected from first week, 10 from second week) of tadpoles used in predation trials. Tadpoles in trial tanks were fed 10% of their biomass of rabbit chow per day. We added two *Anax* to each trial tank 24 h after tadpoles were moved into trial tanks, thereby initiating the predation trials. After another 24 h, all tadpoles were removed from trial tanks and were counted, euthanized, and preserved. Mortality in trial tanks after the 24 h trial was used to estimate predation rate. Each treatment combination was replicated seven times, with one additional set of tanks used to estimate survival of the 40 tadpoles in the absence of predators. Predation rate during trials performed during weeks 1 and 2 and log-transformed tank means for mass and infection of tadpoles on each date were analyzed using repeated-measures ANOVA. The remaining tadpoles in exposure tanks were collected on July 14 and 15, and log-transformed final survival was analyzed using ANOVA.

Experiment 4: Density-dependent infection rates

To evaluate the effects of density on infection rates (Mechanism C), we performed a mesocosm experiment in which tadpoles were exposed to parasites at a range of densities. Density depends on both the number of animals and spatial scale, so we manipulated both factors here. We performed a 3 x 8 factorial mesocosm experiment in which we manipulated both the initial number of tadpoles per container (25, 50, 75, 100, 125, 150, 200, and 250 tadpoles) and spatial scale (i.e., container size: 100 L [diameter x depth = 90 x 20 cm], 300 L [120 x 30 cm], and 1,300 L [150 x 75 cm]). We had one missing treatment (300 L x 75 tadpoles). We also had 6 additional containers to assess tadpole survival in the absence of parasites: two containers of each size, stocked with either 50 or 250 animals. All containers were filled with aged well water and ~100 mg leaf litter. On July 29, 2011, tadpoles (17.5 ± 1.3 mg, originating from 14 egg masses [collected between June 28 and July 1, 2011]) were moved into containers. Three infected snails were then added in cages to each treatment container. Tadpoles were fed 10% of their biomass per day with 3:1 Purina® Rabbit Chow: TetraMin® Fish Flake mixture on day 1 and 4 of the six day experiment.

Because temperature can influence the rate at which snails shed cercariae (Morley et al. 2010), we also assessed temperature differences between different sized containers. HOBO pendant (UA-001-64) temperature loggers were placed in one container of each size to measure water temperature over a 24 h period (beginning at 12:00pm on August 3). The loggers were

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suspended with weights from a floating piece of polystyrene 6 cm from the water surface. On August 6, the experiment was terminated and all animals were collected, euthanized, and preserved in 70% ethanol. Ten tadpoles were haphazardly selected from each of the containers and dissected to measure infection loads. Log-transformed survival and tank mean infection intensity were analyzed using linear models. AIC values were calculated for all permutations of both predictors and the interaction to determine which terms to include in the final models.

Experiment 5 and 6: Effects of predator cue on parasite susceptibility

To evaluate the effect of predator cue on infection rates and survival after parasite exposure (Mechanism D), we performed two experiments in 8 L aquaria using controlled exposures to cercariae and a gradient of predator cue concentrations. For both experiments, cercariae were collected from infected snails placed ~10 cm beneath a 60 W light. We counted cercariae in a watch glass under a dissecting microscope and moved into plastic cups containing 60 mL water, and all cercariae were introduced to tadpole hosts within 8 h of leaving snail hosts.

Experiment 5 examined the effects of predator cue on infection rates. We used a 3 x 2 factorial design with 10 replicates in which we exposed green frog larvae to three predator cue concentrations (none, low, or high) and 0 or 200 echinostome cercariae. On the morning of August 17, 2011, five tadpoles (23.8 ± 1.1 mg, originating from four egg masses [collected on July 26]) were moved into each aquarium and allowed 1 h to acclimate. To produce predator cues, two sets of seven *Anax* were placed in plastic cups containing 0.5 L of water and fed either 100 mg or 300 mg green frog larvae to generate the low and high cue treatments, respectively. The water from containers for each treatment was then mixed together in a bucket and divided evenly among the aquaria at each cue level (~175 mL cue per aquaria). Predator cue was added to aquaria 2 h after tadpoles were added; water was used for the no cue treatment. We added 0 or 200 cercariae in 60 mL water to aquaria 1 h after predator cue addition. We performed behavior observations 15 min after addition of cercariae by slowly approaching aquaria and counting the number of individuals that were active over a 5 s interval. We performed 10 sets of observations (~9 min per set) over 90 min. After 48 h, all tadpoles were collected, euthanized, preserved in 70% ethanol, and later dissected to measure infection loads. Log-transformed infection intensity (tank mean) and arcsine-square root transformed activity (mean proportion active) were analyzed using ANOVA.

Experiment 6 examined how predator cue concentration affects the survival of tadpoles after parasite exposure (i.e., removed from any effect of cue on initial infection intensity). We used a 13 x 3 factorial design varying predator cue concentrations (none, low, and high) and parasite exposure levels (0, 5, 10, 20, 25, 30, 35, 40, 45, 50, 55, 60, or 65 cercariae per individual). Each parasite exposure level was crossed once with each predator cue concentration level, with the exception that we had two sets of the 20 cercariae treatment. On July 19, 2011, tadpoles (22.7 ± 1.2 mg, originating from 14 egg masses [collected on June 28 and July 1]) were moved into the laboratory and allowed to acclimate 1 h prior to beginning parasite exposure. Tadpoles were then added individually to plastic cups containing 60 mL of water and the appropriate number of cercariae. Tadpoles were left in the cups overnight (12-18 h) to ensure exposure, and then five tadpoles were moved to the aquaria of appropriate treatments on the following morning. Aquaria contained either an empty cage or a caged *Anax*. Predators in the low and high treatments were fed 100 mg and 300 mg tadpoles three times per week during the two week experiment. We changed the water in aquaria after one week. Behavior observations were performed 72 h after parasite exposure. The number of active tadpoles was counted for each aquarium 10 times over 90 min, as in Experiment 5. We measured survival over the two weeks and final mass at the end of the experiment. Final survival was analyzed using ordinal logistic regression, and log-transformed final mass and arcsine-square root transformed activity were analyzed using linear models. We did not measure infection intensity in this experiment, but parasite exposure level is strongly correlated with infection load using the method employed here (Marino, unpublished data).

Parasite Identification to Species

One infected snail from each pond was used for species-level parasite identification. Infected snails used for identification were preserved in 70% alcohol for later dissection. Five parasite larval stages (sporocysts or rediae) were dissected from snails and DNA was extracted using a Qiagen DNeasy Tissue Extraction Kit. DNA samples were run through PCR with the digenean-specific primers Dig12 and 1500R (used in Tkach et al. 2000, Olson et al. 2003). The PCR product was run through gel electrophoresis and purified using a Qiagen QIAquick Gel Extraction Kit, and the product was submitted to the University of Michigan DNA Sequencing Core for sequencing. Chromatograms for each sequence were examined in FinchTV version 1.4,

and clean sequences were compared to those of known species using the NCBI Nucleotide BLAST algorithm (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Results

Experiment 1: Combined effects of echinostomes and predators

Compared to controls, addition of caged predators decreased survival 8%, and addition of free predators decreased survival 62% (Figure 3.1a). Addition of parasites decreased survival 10% compared to controls, 42% when combined with caged predators, and 91% when combined with free predators (Figure 1a). In the no vs. caged predator comparison, the negative effect of caged predators on survival was significant for both dates, the negative effect of parasites on survival was significant only for the second date, and the interaction was not significant on either date (Table 3.1). In the no vs. free predator comparison, the negative effects of predators and parasites were significant on both dates, and the interaction was significant on both dates, suggesting a synergistic effect on survival. Finally, in the caged vs. free predator comparison, there was significantly lower survival of tadpoles exposed to free predators compared to those exposed to caged predators on both dates, the negative effect of parasites on survival was significant on both dates, and the interaction was significant mid-experiment but marginally non-significant on the final date. The significant interaction in the caged vs. free predator comparison suggests that the combination of free predators and parasites had a greater interactive effect than the combination of caged predators and parasites. However, by the end of the experiment, the survival of tadpoles in the presence of free predators was reduced to such an extent that the interaction was more difficult to detect and marginally non-significant. The block effects were not significant for any comparison.

Parasites had a significant positive effect on activity levels (Figure 3.1b; $F_{(1, 12)} = 9.30$, $p = 0.01$), predators had a marginally non-significant negative effect ($F_{(1, 12)} = 4.17$, $p = 0.06$), and the predator x parasite interaction was marginally non-significant ($F_{(1, 12)} = 4.50$, $p = 0.06$). The MANOVA of final mass and Gosner stage revealed no significant effect of predators (Wilks' $\lambda = 0.67$, df = 2, 20, p = 0.09), parasite exposure (Wilks' $\lambda = 0.65$, df = 1, 20, p = 0.8), or the predator x parasite interaction (Wilks' $\lambda = 0.65$, df = 2, 20, p = 0.8). Final infection intensity did not differ among predator treatments ($F_{(2, 8)} = 1.51$, $p = 0.3$) or across dates ($F_{(1, 12)} = 1.61$, $p = 0.2$), and the predator x date interaction was not significant ($F_{(2, 12)} = 1.691$, $p =$

0.23); however, the block effect was significant (F (4, 8) = 6.18, p = 0.01). Final infection intensity of the caged tadpoles were much lower (mean ± SE = 1.1 ± 0.4 metacercariae per individual) than free animals (19.4 ± 1.7 metacercariae per individual) and also did not differ among predator treatments (F (2, 7) = 2.325, p = 0.2). Predator and parasite treatments also did not affect survival or final mass of animals in cages.

Experiment 2: *Effects of echinostome exposure on predation risk*

The results of the survival analysis demonstrated that the first predation event occurred more quickly in aquaria that contained infected snails (z=2.08, p=0.04). Block effects were also significant (z=-2.006, p=0.04). Overall predation rates on tadpoles exposed to parasites were higher than controls, with the greatest difference occurring 150 min after the addition of predators, with 17% lower survival in treatments with infected snails (Figure 3.2). All tadpole mortality appeared to be due to predation during the experiment, because animals were either consumed or visibly damaged by predators. One infected snail was consumed by the *Anax*, but excluding that aquarium did not affect results.

Experiment 3: Effects of echinostome infection on predation risk

Prior exposure to echinostomes and predator cue did not affect predation rates in the mesocosm predator trials (p > 0.1), but predation rate decreased between week 1 and 2 (F (1, 20) = 24.85, p < 0.001). Mean infection intensity of tadpoles from exposure tanks was not affected by predator treatment (F (1, 7) = 0.012, p = 0.91) or block (F (7, 7) = 1.044, p = 0.48), and the date x predator treatment interaction was not significant (F (1, 14) = 0.882, p = 0.36). Mean size of tadpoles from exposure tanks increased between weeks 1 and 2 (F (1, 28) = 0.58, p <0.001) but was not affected by predator treatment (F (1, 21) = 0.012, p = 0.92), parasite treatment (F (1, 21) = 2.14, p = 0.16), the parasite x predator interaction (F (1, 21) = 2.06, p = 0.17), or block effects (F (7, 21) = 1.24, p = 0.33). As in the no vs. caged predator comparison in Experiment 1, survival in exposure tanks decreased in the presence of caged predators (F (1, 21) = 5.56, p = 0.007) and infected snails (F (1, 21) = 8.85, p = 0.007), but there was no evidence for a predator x parasite interaction (F (1, 21) = 0.56, p = 0.46) or block effect (F (7, 21) = 1.00, p = 0.46).

Experiment 4: Density-dependent infection rates

The initial number of animals per container and the number x container size interaction did not explain any variation in final infection intensity or survival and was excluded from the final regression models based on AIC. Log infection intensity was negatively correlated with log

container size (Figure 3.33; b = -0.26, t (20) =4.948, p = 0.02), and log container size explained a significant portion of the variance (R^2 = 0.25, F (1, 21) = 7.05, p = 0.02). Mean survival was 62% in both control containers and containers containing infected snails. A marginally non-significant trend suggests that survival was lower in smaller containers (F (1, 21) = 4.323, p = 0.050). Mean ± SE temperatures in the 100 L, 300 L, and 1300 L containers were 25.2 ± 0.18 °C, 25.2 ± 0.11 °C, and 25.6 ± 0.08 °C, and maximum daily temperatures were 27.9 °C, 26.9 °C, and 26.6 °C respectively.

Experiment 5 and 6: Effects of predator cue on parasite susceptibility

In experiment 5, infection rates did not differ among predator treatments (F (1, 18) = 0.764, p = 0.4) and the block effect was not significant (F (9, 18) = 0.91, p = 0.5). Activity levels were very low (<1%) across treatments and did not change in response to predator cue (F (1, 47) = 0.87, p = 0.4), parasite treatment (F (1, 47) = 0.074, p = 0.79), the predator x parasite interaction (F (1, 47) = 0.14, p=0.7), or block (F (9, 47) = 1.86, p = 0.08).

In experiment 6, survival decreased with parasite exposure level (z = -2.241, p=0.02), but there was no effect of predator cue (z = -0.214, p = 0.8) or evidence of an interaction (z=0.619, p = 0.5). Final mass was not affected by parasite exposure (t = 1.01, p = 0.3) or predator cue level (t = -0.40, p = 0.7). Activity decreased at higher levels of predator cue (t = -2.82, p = 0.008), but was not affected by parasite exposure level (t = 1.635, p = 0.1).

Parasite Identification

Echinostomes dissected from snails from four ponds (Kaiser South, Duck Pond, West Marsh Dam Pond, and East Marsh) were identified as *Echinostoma revolutum* based on comparison of our sequence in NCBI Nucleotide BLAST (99% similarity, accession AY222246). Echinostomes from the snail from West Marsh #11 were identified as *Echinoparyphium rubrum* (100% similarity, accession JF820595). All experiments except Experiment 2 used snails only where *E. revolutum* was found. Snails used in Experiment 2 were from all five ponds, including eight snails from West Marsh #11; consequently, a mixture of snails infected with either *E. revolutum* or *E. rubrum* was used in Experiment 2. Because parasites were not identified from all snails used, a mixture of echinostome species may have been used in other experiments if *E. rubrum* and *E. revolutum* co-occurred in some ponds.

Discussion

Our results demonstrate that the joint presence of predators and parasites had strong non-additive effects on survival of anuran larvae. As expected, both parasites and free predators decreased larval green frog survival, but together their synergistic effect amplified this mortality by 21%. Importantly, we conducted a series of follow-up experiments to isolate the mechanistic basis of this interaction. Our results directly support our second hypothesis that free predators and parasites have synergistic effects, which implies that the combined effects of predators and parasites may have complex consequences for amphibian demographic processes, because infection intensities here fall well within the range observed in the field (Skelly et al. 2006, Marino and M.P. Holland, unpublished data).

Our results suggest that the effect of parasite-avoidance behavior on predation risk (Mechanism A) contributes to the observed interaction. The observed increase in activity in the presence of infected snails in Experiment 1, even when predator cues were present, suggests that tadpoles increased activity to avoid cercariae, which in turn likely increased susceptibility to predators. The results of Experiment 2 demonstrate that predation rate increased in the presence of parasites, reinforcing this interpretation. This mechanism therefore is driven by a fundamental difference in the behavioral response of larval frogs to parasites as opposed to predators. Increased activity (i.e., avoidance behavior) of larval frogs in response to cercariae enhances the ability of larval frogs to avoid infection by trematode cercariae (Koprivnikar et al. 2006, Rohr et al. 2009, Daly and Johnson 2011). However, increased activity also can increase larval frog susceptibility to visual predators (Anholt and Werner 1998). This tradeoff between susceptibility to parasites and predators is the most likely explanation for the observed interaction demonstrated here. Such a tradeoff also likely contributes to the interactive effects of predaceous salamanders and trematode parasites on larval wood frogs (Belden and Wojdak 2011) and the positive effects of fish predators on infection (Thiemann and Wassersug 2000, Szuroczki and Richardson 2012).

The observed interaction here may result from constraints on the ability of tadpoles to reliably assess risks from the combination of natural enemies. The engagement of tadpoles in parasite avoidance behavior despite the presence of predator cue is surprising, because the individual fitness cost of a predation event outweighs any sublethal costs of parasitism. Here, tadpoles may have perceived greater risk through tactile cues of cercariae than from predator cue. This response may not necessarily be maladaptive, if the immediate benefits of an avoidance

response (i.e., escape from cercariae) outweigh the risk of increased visibility in a natural setting, especially for smaller tadpoles which may be generally less visible and experience greater susceptibility to parasites (Holland et al., 2007). Alternatively, tadpoles may be engaging in a generalized reflexive response (i.e., a physiological response) to irritation, which is maladaptive in the presence of predators. Future research varying the force of infection and perceived threats from predators could provide more insight into the limitations on tadpoles' responses to each threat.

A predation risk – parasite susceptibility tradeoff likely occurs in other systems as well (e.g., Rutherford et al. 2007), because many traits play an important role in susceptibility to parasitism (reviewed in Hart 1990, Moore 2002) as well as predation (e.g., Biro et al. 2003, Strobbe et al. 2011), and these two threats can pose conflicting pressures when the optimal response to predation risk differs from that to parasitism. From an ecological perspective, the consequences of this tradeoff may be elevated parasite transmission or higher predation rates, depending on the perceived fitness costs associated with each natural enemy. Such effects may synergistically alter disease prevalence and population dynamics. From an evolutionary perspective, this tradeoff could create an external constraint on the evolution of phenotypic plasticity in response to natural enemies (i.e., induced defenses), leading to traits which may appear to be maladaptive in the context of a single species-pair interaction.

Our results also suggest that alternative mechanisms (B-D), although likely important in other contexts or systems, made small or no contribution to the observed interaction here. We expected that infected individuals would experience morbidity and thus would be less able to escape from predators (Mechanism B). The results of Experiment 3, however, suggest that parasite infection did not influence predation susceptibility. It is possible that an effect of infection on escape ability, if present, may have been countered by lowered overall activity of more infected individuals after infection (observed in Thiemann and Wassersug 2000) and thus reduced visibility to predators.

If per-capita infection rates increased at lower densities, and parasite-induced mortality increases nonlinearly with infection rates (i.e., mortality occurs only at high infection intensities), predator-induced reduction in prey density could magnify effects of parasites on survival (Mechanism C). However, the lack of an effect of lethal predators on final infection intensity in Experiment 1 and the results of Experiment 4 suggest that predators did not drive higher per-

capita infection rates through effects on tadpole density. A 10-fold increase in density in Experiment 4 at the scale of Experiment 1 (i.e., 1,300 L cattle tank), did not influence final infection intensity, suggesting infection rates are not limited by the number of tadpole hosts at this scale. Interestingly, the significant effect of container size on final infection intensity suggests that infection rates can depend on the scale of host-parasite interaction, which will be important to extrapolate effects measured in the laboratory to larger scales. An explanation for this result is that the change in spatial scale increases the contact rate between parasites and hosts and thus infection rates. Temperature also varied across container sizes, but the relatively small mean difference (< 0.5° C) does not likely entirely explain the observed pattern.

Notably, caged predators had a significant effect on survival in mesocosms (Experiments 1 and 3) – a nonconsumptive effect that has been previously reported in this system (Werner and Anholt 1996, see also McCauley et al. 2011 for a case with odonate larvae). However, contrary to our first hypothesis, the combination of caged predators with echinostomes had additive, rather than synergistic, effects on survival. This finding was consistent with the findings of Raffel et al. (2010), who found that the effects of *Echinostoma trivolvis* and caged newt predators (*Notophthalmus viridescens*) on larval American toads (*Bufo americanus*) were additive. The results of Experiment 1 were insufficient to rule out the contributions of predator cue to the observed interaction entirely (i.e., Mechanism D), because predator cues generated by free predators can exceed those of caged predators (Peacor and Werner 2001). However, the results of Experiments 5 and 6 provide further support that higher cue concentrations did not explain the observed interaction in Experiment 1, because predator cue did not influence infection rates or post-exposure effects of parasites on hosts.

These results contrast with the findings of others, potentially due to differences in design (e.g., predator species, experiment duration, spatial scale, and parasite exposure level). The lack of an effect of parasites or predator cues on activity levels in Experiment 5 was surprising, given demonstrations of such effects elsewhere (e.g., Relyea 2001a, Rohr et al. 2009). Additionally, we failed to observe the positive effect of predator cue on individual infection intensity documented elsewhere (Thiemann and Wassersug 2000, Szuroczki and Richardson 2012). A possible explanation is that we used a relatively small size class (~23 mg), which exhibited a low baseline activity level, so behavioral effects of natural enemies may have been difficult to observe. For our investigation, however, effects on smaller size classes are more relevant, as effects of

infection on survival decline in larger tadpoles (Holland et al. 2007). Finally, although we did not observe interactive effects of echinostomes and predator cue on survival in Experiment 6, Koprivnikar (2010) reported an interactive effect of caged predators and echinostome infection on larval leopard frog (*Rana pipiens*) survival. However, this interaction may have resulted partly from an unexplained positive effect of echinostomes on leopard frog survival in the absence of predators. Additionally, differences among species in parasite susceptibility (e.g., Johnson et al. 2012) may explain why the same interaction did not occur here.

One final mechanism may be important in other systems which we did not address here. Nonlinearities in predator-prey interactions (e.g., type II functional response, Holling 1959) could also drive non-additive effects and can be altered by parasites (Dick et al. 2010). However, given the relatively small effect of echinostomes on survival in the absence of predators in Experiment 1, this mechanism did not likely contribute to the observed interaction.

Given the ubiquity of parasitism as a lifestyle (Dobson et al. 2008) and the dominance of food web links by parasites (Lafferty et al. 2008), measuring the overall effects of parasites will be essential to developing predictive models of trophic interactions within many animal communities. Synergistic effects of predators and parasites provide additional challenges to the already difficult task of measuring interaction strengths. However, our approach of separately evaluating potential mechanisms provides a powerful method to determine which processes are dominant or unimportant. In particular, trait-mediated tradeoffs in susceptibility are likely drivers of potential synergisms and merit greater attention. Such tradeoffs may be mediated by behavior (as evidenced here), by physiology (Ramirez and Snyder 2009), or by other traits (e.g., growth, Duffy et al. 2011). The resulting synergism could modify important ecological processes, such as dilution effects, trophic cascades and keystone effects.

In addition to furthering our knowledge of the role of multiple natural enemies in animal communities, these results have important implications for amphibian conservation and wetland management, as echinostomes have been reported to be in higher abundance near human activities, such as pesticide use (Rohr et al. 2008) and urbanization (Skelly et al. 2006). A realistic evaluation of the impacts of higher parasite abundance must include the influence of existing stressors of amphibians, which typically include predators. More generally, these results can inform our understanding of interactions among multiple stressors on amphibian populations, which is of particular importance due to recent global amphibian declines (Stuart et al. 2004).

Acknowledgements

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Table 3.1: Results of ANOVAs for comparisons of survival across predator and parasite treatments on day 17 and day 26 of Experiment 1. The three predator treatments were no predator, 2 caged *Anax* predators, and 2 free *Anax*. Survival was log transformed prior to analysis.

Estimated Survival - Day 17

No vs. Caged Predator	F	df (between groups)	df (within groups)	p
Predator	7.07	1	12	**0.02***
Parasite	1.35	1	12	0.3
Predator x Parasite	0.62	1	12	0.6
Block	0.65	4	12	0.4
No vs. Free Predator				
Predator	63.72	1	12	**<0.001***
Parasite	12.96	1	12	**0.004**
Predator x Parasite	10.49	1	12	**0.007**
Block	1.84	4	12	0.2
Caged vs. Free Predator				
Predator	4.93	1	12	**<0.001***
Parasite	3.19	1	12	**0.002**
Predator x Parasite	1.03	1	12	**0.04***
Block	1.42	4	12	0.2

Final Survival

No vs. Caged Predator	F	df (between groups)	df (within groups)	P
Predator	7.27	1	12	**0.02***
Parasite	11.05	1	12	**0.006**
Predator x Parasite	3.457	1	12	0.09
Block	0.952	4	12	0.5
No vs. Free Predator				
Predator	38.95	1	12	**<0.001***
Parasite	9.37	1	12	**0.01**
Predator x Parasite	6.79	1	12	**0.02***
Block	1.12	4	12	0.4
Caged vs. Free Predator				
Predator	24.90	1	12	**<0.001***
Parasite	11.32	1	12	**0.006**
Predator x Parasite	3.50	1	12	0.09
Block	11.32	4	12	0.6

Significance codes: '***' 0.001, '**' 0.01, '*' 0.05
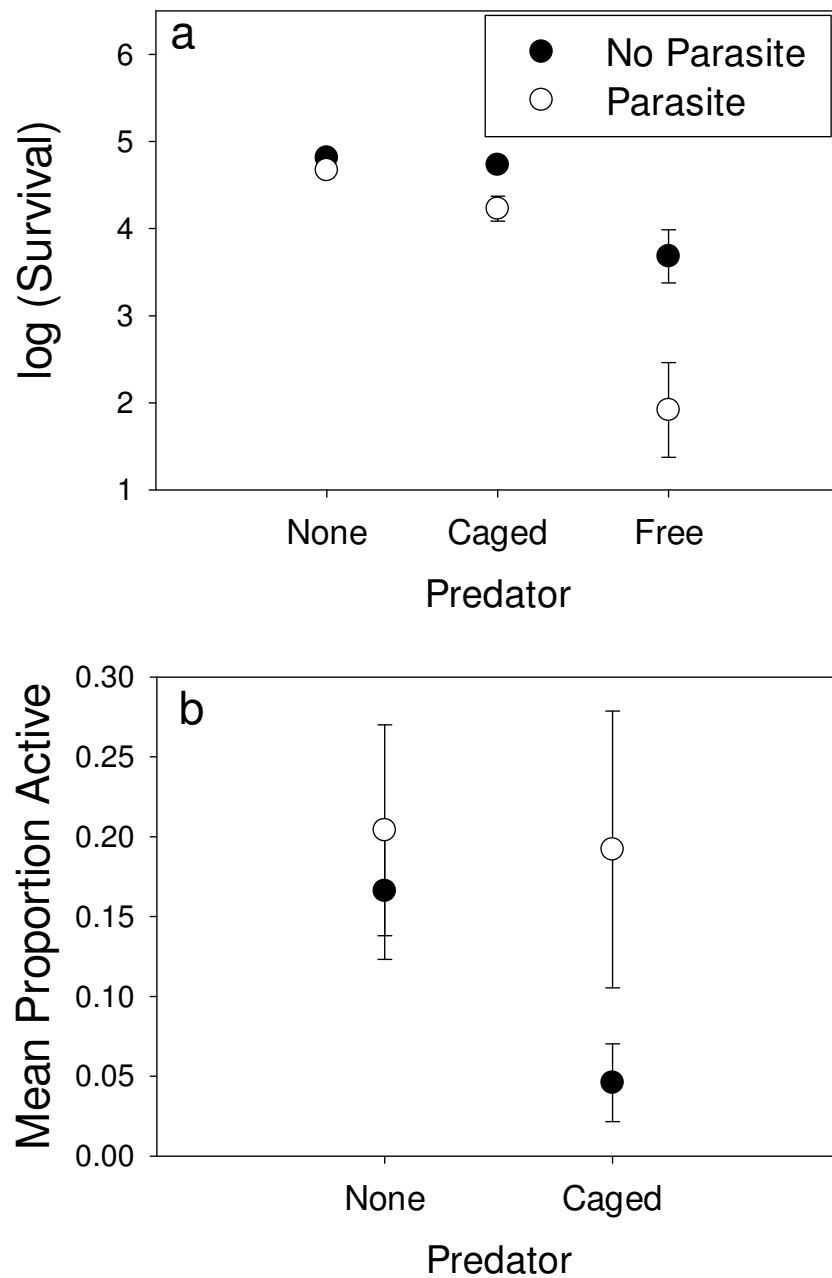
Figure 3.1: Results of Experiment 1: a) Log number of surviving green frog larvae (mean ± SE) after 26 days in the presence of no predator, two caged (nonlethal) *Anax* predators, or two free (lethal) *Anax* and in the absence (black circles) or presence (white circles) of echinostome parasites. b) Proportion of visible tadpoles (mean ± SE) that were active in the no predator and caged predator treatments in the presence and absence of echinostome parasites.
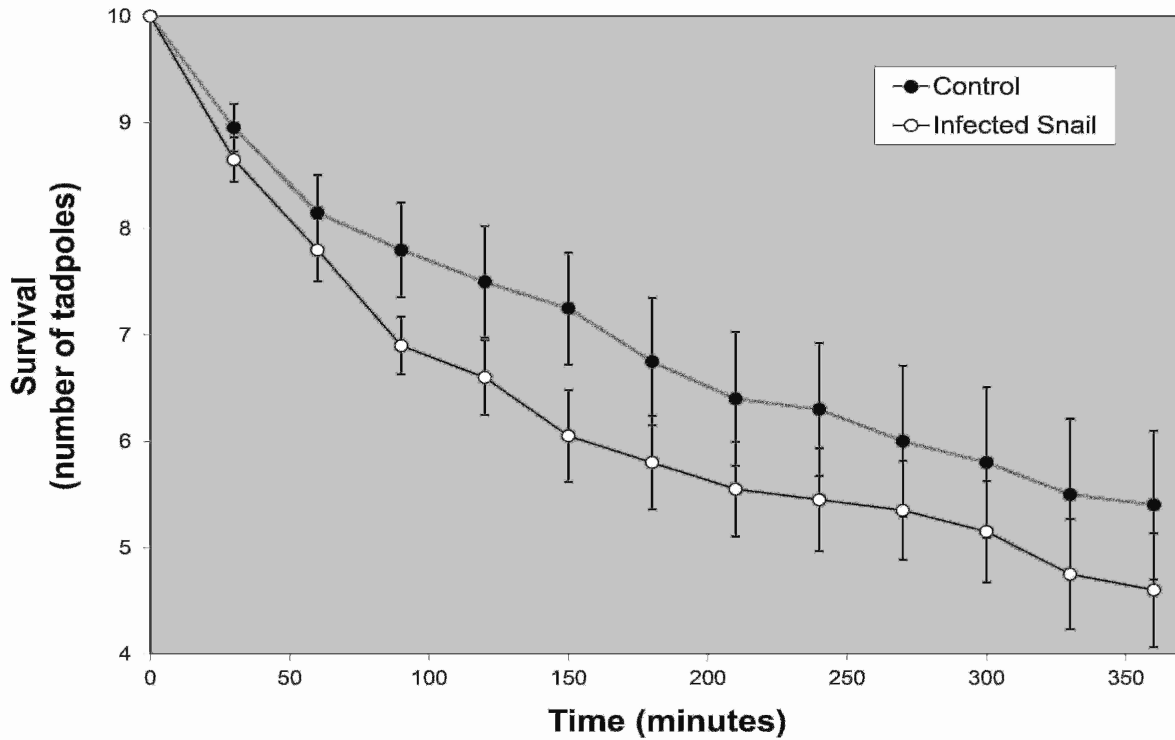
Figure 3.2: Number of surviving larval green frogs (mean ± SE) over 6 h predation trial in aquaria during Experiment 2, during which tadpoles were exposed to one *Anax* predator. Trials began with 10 tadpoles in each aquarium and included one uninfected (closed circles) or infected (open circles) *Planorbella trivolvis* snail.
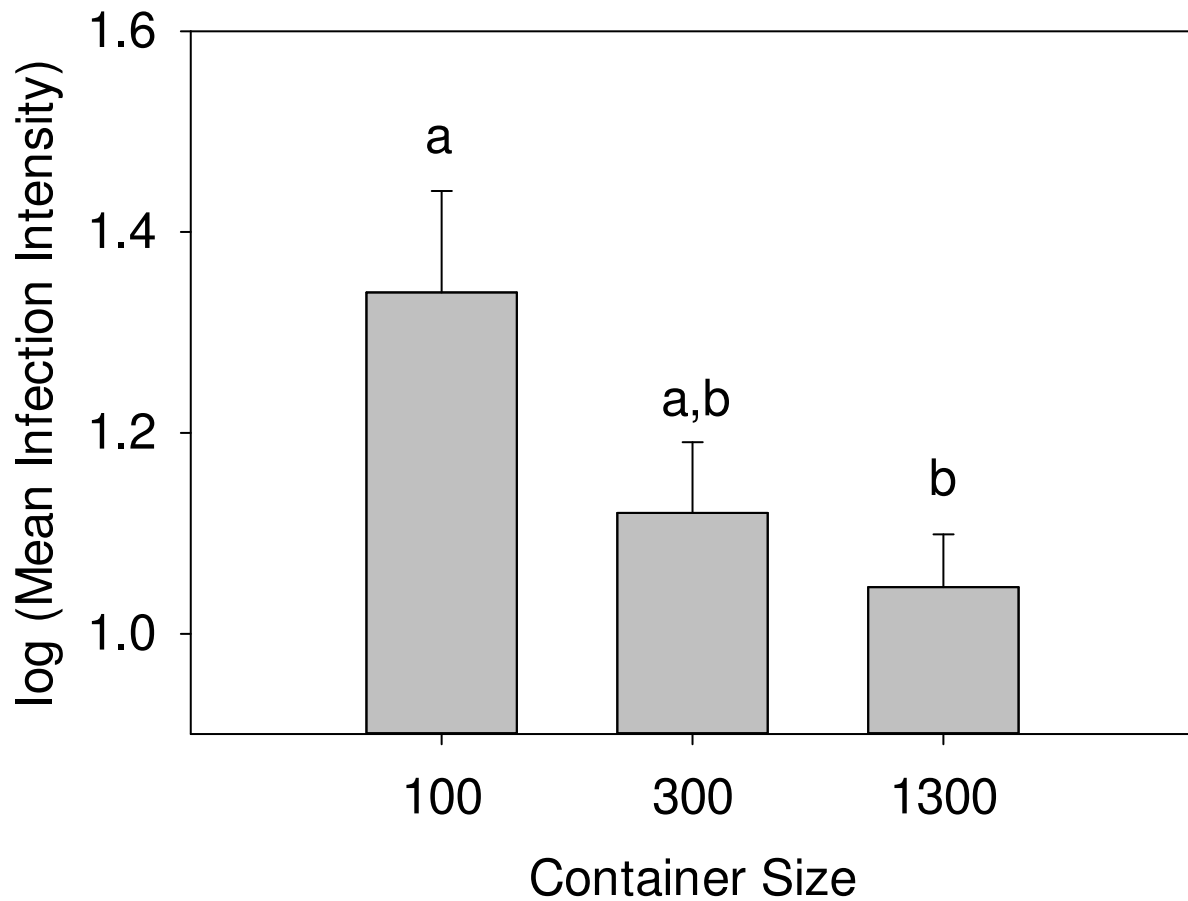
Figure 3.3: Log individual infection intensity (mean ± SE number of metacercariae) of tadpoles in 100 L, 300 L, and 1300 L containers in Experiment 4. Different letters indicate significant differences between sizes (Tukey HSD, $\alpha = 0.05$). The number of tadpoles per container ranged from 25 to 250, but the number of tadpoles did not affect infection intensities ($F(1, 20) = 0.12$, $p = 0.7$).

Chapter IV

Interactive effects of parasitism and competition on larval anuran growth and survival

Introduction

  There is a considerable literature on the impact of parasites on the performance or traits of individual organisms (e.g., Scott 1988, Barber 2000, Johnson et al. 2012). However, scaling these effects up to consequences to population dynamics and interactions among species requires that we understand (or have functional relationships regarding) how these impacts change with ecological context of that individual. For example, there is evidence to suggest that species' density (Steinhaus 1958, Begon 2008, Johnson et al. 2013), the intensity of competition (Barnes and Siva-Jothy 2000, Bedhomme et al. 2005, Koprivnikar et al. 2008), and the size of the organisms (McDonald et al. 2006, Holland et al. 2007, Hechinger 2013) all can have important effects on the infection rates and impact of parasites on individuals. Host density especially merits attention because of its commonly central role in mediating parasite transmission (McCallum et al. 2001, Begon 2008). However, the direct effects of density on host-parasite interactions cannot often easily be examined in isolation, because the strength of competition also depends directly on density, and competition can affect susceptibility to parasitism (e.g., through reduced nutrition, Coop and Kyriazakis 1999, Smith et al. 2005). Furthermore, the separate and joint effects of parasites and competition on individual hosts are unlikely to be uniform within populations, due to trait variation. For instance, host size structure can influence and be influenced by interactions with parasites (Holland et al. 2007, Clague et al. 2011) and competitors (Persson 1983, Morin and Johnson 1988), with potential consequences of host heterogeneity for species interactions (e.g., trait-mediated indirect effects, Werner and Peacor 2003) and population dynamics (Lomnicki 1978, Dwyer et al. 1997).

Here, we examine the influence of parasitism, host density, and host variation (size structure) in a simple aquatic community. We examine the separate and joint effects of trematode parasites (Digenea: Echinostomatidae) and competition on two size classes of larval anurans (large and small green frogs [*Rana clamitans*]) that differ in susceptibility to parasites. We hypothesized that increased small tadpole density would indirectly reduce infection rates in both size classes of tadpoles, due to a reduction in the ratio of parasite infective stages (cercariae) to hosts. We expected a decrease in this ratio because echinostomes are indirectly transmitted, so that the number of cercariae present does not increase with local tadpole host density, at least over short timescales. We also hypothesized that parasites would indirectly benefit larger tadpoles in competitive interactions, because size confers increased tolerance of infection (Holland et al. 2007), resulting in density- and trait-mediated indirect effects of parasites. To test these hypotheses, we examined the separate and combined effects of parasitism and host density on two size classes in two mesocosm experiments. We then coupled the results of these experiments with findings from two similar previous experiments to examine the generality of observed effects.

Study System

Echinostomes have a complex life cycle involving a snail first intermediate host, an amphibian, fish, or mollusk second intermediate host, and a bird or mammal definitive host (Kanev et al. 2000). Within the snail first intermediate host, the parasite undergoes multiple rounds of asexual reproduction during sporocyst and redia stages before producing a free swimming infective stage, cercaria, which then enters the second intermediate host. In amphibians, cercariae enter through the cloaca and migrate to the kidneys, where they encyst, forming metacercariae (Najarian 1954). Echinostomes have a range of effects on amphibian hosts, such as reduced growth rates, impaired kidney function and death at high infection intensities (Fried et al. 1997, Holland et al. 2007, Marino et al., in press). Larger tadpoles at later developmental stages are less affected by infection (Schotthoefer et al. 2003, Holland et al. 2007). Green frogs, our focal species, have a long (~3 month) breeding season and often overwinter as tadpoles, so that tadpoles of different size classes frequently co-occur.

Methods

General notes on animal collection and care

Green frog egg masses were collected from the experimental ponds on the Edwin S. George Reserve (ESGR) in Livingston County, MI, and placed in 300 L wading pools filled with aged well water. After hatching, tadpoles were fed Purina® Rabbit chow ad libitum until the beginning of experiments. Mesocosms used in experiments and to culture large green frog tadpoles were 1,300 L cattle tanks (150 cm diameter x 75 cm depth) filled with aged well water, covered with 60% shade cloth and located in an open field. To each tank, we added ~300 g leaf litter (mostly *Quercus*) as a substrate, zooplankton and phytoplankton inocula (the latter as a resource for tadpoles), and 25 g of Purina ® Rabbit Chow to provide an initial source of food and nutrients.

Planorbella trivolvis snails (~1 g) were collected from three ponds in Livingston County, MI. Snails were screened for trematode infection by placing them in 60 mL water in cups under a 60 W light. After 4 h, all cups were examined for the presence of trematode cercariae under a dissecting microscope. A few cercariae from each snail were then placed in 70% ethanol and identified as echinostomes after Schell (1985). Echinostomes in snails from these ponds were previously identified as *Echinostoma revolutum* using molecular methods (ponds referred to as Duck Pond [42.481308, -83.983442], Kaiser South Pond [42.430299, -84.036582], and East Marsh [42.45679, -83.996748] in Marino and Werner, in press), and we expect that we used the same species here. This research was performed in accordance with University of Michigan UCUCA Protocol #07765.

Experiment 1: Parasitism in two size classes across a host density gradient

We performed an experiment in mesocosms to test the effects of parasites on two size classes of hosts across a density gradient. We also included predator presence as a factor in this experiment, because laboratory experiments suggest that predators and parasites can have interactive effects on tadpoles (Thiemann and Wassersug 2000, Szuroczki and Richardson 2012, Chapter II). We followed a 3 x 2 x 2 factorial, randomized block design with five replicates. Each mesocosm contained five large green frog tadpoles (LG) and 0, 50, or 100 small green frog tadpoles (SG), three uninfected or infected *P. trivolvis* snails, and two empty cages or two caged odonate predators. The densities and parasite exposure levels here fall well within the ranges observed in natural populations (Skelly et al. 2006, Werner et al., unpublished data, Chapter

VII). Predators were late-instar larval *Anax junius* or *A. longipes*, common odonate predators of larval frogs in eastern North America, collected from the ESGR experimental ponds.

LG were reared from eight egg masses collected on June 8 and 10, 2011. After 3 weeks, 600 tadpoles from these masses were moved from 300 L culture pools and divided equally among three 1,300 L mesocosms. Two additional mesocosms were set up after an additional 2 weeks, each containing 150 tadpoles, to ensure that enough LG would be available for the experiment. To encourage growth, an extra 25 g of rabbit chow was added to all tanks on July 18. SG were reared from nine egg masses collected from July 12-15.

Experimental mesocosms were filled with water on July 20-22 and set up with plankton inocula on July 24. To initiate the experiment, we added LG (400-450 mg each) and SG (10-15 mg each) on August 1 and 2, and predators and snails were added to appropriate containers after all tadpoles were added on August 2. Predator cages were constructed from a 10 x 10 cm piece of slotted drain pipe enclosed by window screening fixed with rubber bands. To generate chemical cue, caged predators were fed ~300 mg green frog tadpoles three times per week for the duration of the experiment. The three snails in each container were put into a single cage. After four weeks, the experiment was terminated, all tadpoles were collected, and all five LG from each container and 10 randomly selected SG from the 50 and 100 SG containers were weighed. All tadpoles were then euthanized and preserved in 70% ethanol. To measure infection, two LG and 10 SG were dissected from each container in the parasite treatments. We removed the mesonephri and pronephri and counted the number of echinostome metacercariae present in each kidney and nephric duct after Holland et al (2007).

Experiment 2 – Effects across a broader density gradient

After finding evidence for an interactive effect of parasites and competition on growth in Experiment 1 (see Results), we examined their joint effects across a broader range of tadpole densities. We performed a 2 x 3 factorial mesocosm experiment with five replicates in which we manipulated tadpole density (25, 100, or 200 SG) and the presence or absence of infected snails. Mesocosms again contained 5 LG, but predators were not included as a factor. LG (250-300 mg each, from 6 egg masses collected May 24, 2012) were reared throughout the summer in 300 L pools and fed rabbit chow ad libitum. LG in this experiment were smaller than in Experiment 1, because larger unexposed tadpoles were unavailable. SG (10 -15 mg each) were reared from seven egg masses collected on July 25 and 30, 2012. We filled and added leaf litter to cattle

tanks on July 25. We inoculated tanks with zooplankton and phytoplankton and added Purina®
rabbit chow on July 30. Tadpoles and three caged uninfected or infected *P. trivolvis* snails were
added August 10th. The experiment was terminated after four weeks, at which point we weighed
10 randomly selected SG and all LG. All tadpoles were then euthanized and preserved in 70%
ethanol, and 2 LG and 5 SG were later dissected from each container to measure infection. A
smaller subsample was dissected than in the first experiment because the results of Experiment 1
and a previous experiment (Chapter III) suggested that no difference in infection would occur
across densities (see Results).

Statistical analyses

All analyses were performed in the R statistical package v.2.15 (http://www.r-project.org/). Log-transformed infection intensity (number of metacercariae) and final mass were
analyzed using ANOVA. Survival was analyzed using generalized linear mixed effects models
with a binomial distribution. The relationships between infection intensity and final density were
analyzed using linear models.

Results

Experiment 1: Effects of parasites and predators on a simple community

In the analysis of final mass, LG final mass decreased with greater SG density and the
parasite x density interaction was significant for both size classes (Figure 4.1, Table 4.1), while
other treatment effects and interactions were not significant. Parasite presence reduced final mass
of LG at higher densities (with slight to no decrease in SG final mass) but increased final mass of
both size classes at lower densities relative to controls. SG and LG survival did not depend on
density, parasite presence, or predator presence, and no interactions were significant ($p > 0.5$). In
tanks exposed to parasites, individual infection intensities of LG (mean $\pm$ SE = 175.6 $\pm$ 14.3
metacercariae) were much higher than SG (29.3 $\pm$ 2.6 metacercariae) (paired t-test, $t = 7.94$, $df =$
19, $p < 0.001$). LG and SG infection did not depend on initial or final density, predator presence,
or the density x predator interaction ($p > 0.2$).

Experiment 2 – Further effects of parasites on competitive interactions

Despite being covered with shade cloth, nine mesocosms in two blocks were colonized
by predaceous libellulid dragonfly larvae (*Leucorrhinia intacta*). The presence of *L. intacta*
strongly reduced survival of SG (binomial GLMM, $p < 0.001$), so we excluded those nine

containers from further analyses. We thus had three or four remaining replicates of each treatment combination. We also note that at the 25 SG density, the smallest one or two of the five LG were indistinguishable from the largest SG in some containers. We therefore used median rather than mean mass for both LG and SG in our analyses and selected tadpoles for dissection (the 2nd and 3rd largest LG) to avoid potential biases due to misclassifying SG and LG individuals.

Median final mass of LG decreased with greater density, and there was a marginally non-significant negative effect of parasites (p = 0.07), but the density x parasite interaction was not significant (Table 4.1). Median final mass of SG decreased with increased density but did not depend on the presence of parasites, and the density x parasite interaction was not significant. In the analysis of SG survival, the density x parasite interaction was significant (likelihood ratio test, X^2 = 14.05, p < 0.001), and the proportional odds of survival were lowest in the presence of parasites at the highest density (Figure 4.2). Survival of LG did not differ among treatments (p > 0.5). Infection intensity was greater in LG (28.3 ± 5.1 metacercariae) than SG (mean ± SE = 8.6 ± 0.9 metacercariae; t = 4.11, df = 10, p = 0.002) and did not depend on initial density for either size class (SG: F (2, 4) = 2.79, p = 0.2; LG: (F (2, 4) = 0.89, p = 0.5). However, mean SG infection intensity was positively correlated with final density (Figure 4.3).

Discussion

Our results suggest that the context of individual host-parasite interactions are important to consider in scaling up the effects of echinostomes. Infection rates and the effects of parasites on host fitness components (growth and survival) depended on individual size and, at least under some circumstances, host density. Changes in infection rates and host tolerance of infection as a result of density-dependent processes and host variation could mediate the dynamical effects of parasitism on host populations (Dwyer et al. 1997, Kauffman and Jules 2006, Lively 2006, Begon 2008).

The significant parasite x density interactions (SG and LG growth in Experiment 1, SG survival in Experiment 2) are consistent with an interactive effect of competition and parasitism on host fitness. Competitive stress can reduce host condition (e.g., due to elevated corticosterone stress hormone levels, Glennemeier and Denver 2002), which may increase host susceptibility to pathogens (Apanius 1998, Belden and Kiesecker 2005, Echaubard et al. 2012), consistent with a

previously reported marginally non-significant interactive effect (p = 0.056) of echinostome infection and competition on northern leopard frog (*Rana pipiens*) growth (Koprivnikar et al. 2008). It is not clear why different fitness components were affected in the two experiments here, but the range of densities employed were different and absolute growth rates were much higher in Experiment 1 (e.g., in controls at the 100 SG density, SG growth rates in Experiment 1 were 8.5 times greater than Experiment 2 in the absence of parasites, t = -6.318, *p* = 0.002). With respect to survival, the higher range of densities used in Experiment 2 compared to Experiment 1 may offer a partial explanation. Competition may amplify effects of parasites on survival only over a certain threshold (i.e., only at the 200 SG density in Experiment 2). With respect to growth, a potential explanation is that environmental conditions may mediate the interaction between competition and parasitism. Despite the similar design, conditions in Experiment 2 were apparently poorer for tadpole growth, potentially due to differences between years in weather (e.g., 2 °C warmer mean water temperature in mesocosms in Experiment 1) and phytoplankton growth. These differences in growth conditions influenced baseline growth rates, which in turn likely influenced interactions with parasites, because host size can influence infection rates and host tolerance of infection (Holland et al. 2007). In addition to potentially mediating growth effects, growth conditions may also have amplified the negative interactive effects of parasitism and competition on survival in Experiment 2.

To further corroborate these findings, we compared the results of these experiments with findings from two additional mesocosm experiments reported elsewhere (referred to as Experiments 1 and 3 in Marino and Werner, in press). These experiments were conducted for different purposes but used a similar design (see Table 4.2 and Appendix). We examined how the effects of parasites on SG survival and growth depended on initial density and absolute growth rate across experiments. Across experiments, the effects of parasites on SG survival became more negative as initial densities increased (Figure 4.4a; QM = 5.66, df = 1, p = 0.017,) but did not depend on absolute growth rates (QM = 0.23, df = 1, p = 0.63). The effects of parasites on SG growth became more positive with higher absolute growth rates (Figure 4.4b; slope = 0.05, QM = 5.45, df = 1, p = 0.020) but did not depend on initial density (QM = 0.041, df = 1, p = 0.84). These results are consistent with the hypothesis that differences in densities used and growth conditions contributed to different parasite effects observed in these experiments.

Our findings exemplify the challenges in scaling up individual host-parasite interactions. Parasites did not substantially decrease SG growth across experiments, despite evidence at smaller scales that parasites have strong negative effects on small green frog tadpole growth at comparable infection intensities (Chapter II). Instead, effects of parasites on SG were near to neutral or positive. The difference between studies probably relates to dynamical changes in and feedbacks between resource levels, infection rates, and densities that were not present in earlier studies at smaller scales. Furthermore, in contrast to SG, a negative effect of parasites on LG occurred under some circumstances (i.e., at the 100 SG density in Experiment 1 and a marginal effect across densities in Experiment 2). LG thus experienced detectable negative effects of parasites under conditions where SG did not, despite evidence that larger tadpoles experience fewer effects of infection under individual exposures in the laboratory (Holland et al. 2007). The much higher infection intensities in LG likely provide an explanation, as effects of echinostomes on growth are intensity-dependent (Chapter II).

A surprising result was that parasites tended to positively affect growth under some conditions, although similar positive effects of helminth parasites on growth have been documented in other systems (Phares 1996, Arnott et al. 2000). For example, infection with the trematode, *Ribeiroia ondatrae*, increases size at metamorphosis of the Oregon spotted frog, *Rana pretiosa* (Johnson et al. 2012). Thinning (i.e., a parasite-induced reduction in density) is unlikely to be responsible here, as parasites did not affect survival in Experiment 1. Instead, a possible explanation is that tadpole hosts adaptively respond to the presence of parasites by increasing growth rates through increased foraging rates or altered metabolism, when environmental conditions allow. Increased growth rates could be adaptive, because the costs of parasitism decrease with size (Schotthoefer et al. 2003, Holland et al. 2007). In the absence of parasites, intrinsic or extrinsic costs associated with accelerated growth rates (e.g., a growth-mortality tradeoff, Schiesari et al. 2006) may restrict tadpole growth. However, in the presence of parasites, growth costs may be outweighed by the risks and costs associated with parasitism. An interactive effect of parasitism and competition may result because an adaptive growth response is only possible when resource levels are sufficient to counteract the costs of infection.

Our results also demonstrate the influence of density on parasite transmission. Density may simultaneously influence infection rates through multiple mechanisms, which may counteract each other: 1) Increased host densities may reduce the ratio of cercariae to hosts (our

first hypothesis). 2) Increased host densities can reduce host size through competition, and larger tadpoles experience higher infection rates (Holland et al. 2007). 3) Increased host densities can reduce host condition (e.g., due to elevated stress hormone levels, Glennemeier and Denver 2002), which may impair parasite resistance (Belden and Kiesecker 2005). Only the third mechanism was directly supported here, because the results of Experiment 2 revealed a positive relationship between final density and infection intensity, consistent with a competitive stress effect on host condition and resistance. This result is also consistent with a positive relationship between density and infection intensity at the mesocosm scale that has been documented in another amphibian-trematode (*R. ondatrae*) system, even though a negative effect of density was found at the aquarium scale in the same study (Johnson et al. 2013). We found no evidence for the first mechanism, because increased initial density did not reduce mean infection intensity, in line with previous studies (Raffel et al. 2010, Marino and Werner, in press). One explanation is that most cercariae are unsuccessful in locating and infecting hosts at the large mesocosm scale, so that increased density has a relatively minor impact on the number of cercariae per host. The second mechanism was also not directly supported, although size did affect infection rate in comparing SG to LG within experiments, which suggests that a negative effect of density on size could affect infection over longer timescales than those addressed here.

With respect to our second hypothesis, despite evidence that size structure influenced host-parasite interactions, we found no support for the predicted density- or trait-mediated indirect effects of parasitism. Direct effects of parasites on LG apparently outweighed any indirect benefit mediated through effects on SG, likely due to the high infection intensities in LG. Several factors may contribute to differences among size classes in infection intensity, including better detection of larger hosts by cercariae, less intraspecific competition among parasites due to more kidney tissue available in larger hosts, and host choice by parasites (e.g., Wojdak et al. 2013). From the parasite perspective, transmission to definitive hosts may be more likely for metacercariae in larger tadpoles, because larger tadpoles are more tolerant of infection than smaller tadpoles (Holland et al. 2007). Larger tadpoles also likely experience lower background mortality (Werner 1986) and may be preferred prey by mammal and avian definitive hosts due to greater visibility and nutritional content. However, the fitness advantages of infecting a larger host are not necessarily greater, as larger tadpoles are also more efficient at eliminating cysts (Holland 2009).

Context (i.e., density and growth conditions) and trait (i.e., size) dependence poses challenges to incorporating parasites into population and community models. Nevertheless, such factors are crucial to consider and merit additional research, as our results suggest that the magnitude and even direction of parasite effects can change, and such interactions are likely common. Many animals tolerate low resource levels in the absence of disease, but the combined effects of competition (e.g., poor nutrition) and parasitism can act synergistically to reduce host fitness (Bedhomme et al. 2004, Sadd 2011, Vale et al. 2011). Moving forward, it will be useful to identify consistent tradeoffs (e.g., resource allocation to parasite defenses vs. other fitness components) and involved traits (e.g., growth rates) that can be used to incorporate competition into broad theory of host-parasite interactions.

Finally, the observed effects may facilitate important interactions at larger ecological scales. First, feedbacks between parasitism, host size structure, and competitive interactions may influence population dynamics and evolution (Peacor et al. 2007, Donnelly et al. 2013). Second, parasite effects on growth and survival may mediate apparent competition and keystone species effects (Hudson and Greenman 1998, Hatcher et al. 2006), comparable to effects of predators (Paine 1966, Werner and Peacor 2003). Third, a positive effect of competition on infection rates mediated through physiology may counteract potential dilution effects, because reduced contact rates caused by higher host densities or altered host community composition may be offset by impaired resistance to infection due to competitive stress. Finally, effects of competition and size structure on parasite transmission and persistence (e.g., due to host death) may also influence transmission to definitive hosts, with potential implications for long-term host-parasite dynamics and community structure. Interactions between competitive and host-parasite interactions may thus have important implications for the relationships between density, host diversity, and disease.

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Table 4.1: ANOVA results for final mass of LG and SG in Experiments 1 (mean mass) and 2 (median mass).

	Experiment 1				Experiment 2			
LG Final Mass (log-transformed)	**F**	**df (between groups)**	**df (within groups)**	**p**	**F**	**df (between groups)**	**df (within groups)**	**p**
Parasite	0.001	1	48	0.97	4.33	1	13	0.066
Density	9.66	1	48	**<0.001**	4.92	1	13	**0.045**
Predator	3.08	1	48	0.086				
Parasite x Density	4.42	1	48	**0.018**	0.054	1	13	0.82
Parasite x Predator	0.81	1	48	0.37				
Predator x Density	2.80	1	48	0.07				
Predator x Parasite x Density	1.43	1	48	0.25				
Block	3.83	4	48	**0.0093**	3.7	4	13	**0.032**
SG Final Mass (log-transformed)								
Parasite	0.44	1	28	0.51	0.83	1	13	0.38
Density	2.22	1	28	0.15	7.65	1	13	**0.016**
Predator	0.25	1	28	0.62				
Parasite x Density	5.68	1	28	**0.024**	0.025	1	13	0.88
Predator x Parasite	2.18	1	28	0.15				
Predator x Density	0.002	1	28	0.96				
Predator x Parasite x Density	0.022	1	28	0.88				
Block	2.56	4	28	0.061	0.65	1	13	0.63

Table 4.2: Summary of four mesocosm experiments that were compared to examine the dependence of parasite effects on growth and survival on density and growth rates.

Experiment	Referred to as:	SG Density	LG Density	Duration	Replicates	Infection (mean ± SE metacercariae)
A	Experiment 1 in Marino and Werner, in press	200	0	26 d	5	19.4 ± 1.7
B	Experiment 3 in Marino and Werner, in press	250	0	14 d	8	41 ± 9.4
C	Experiment 1 here	0, 50, 100	5	28 d	5	30.15 ± 3.5
D	Experiment 2 here	25, 100, 200	5	28 d	3-4	8.6 ± 0.9
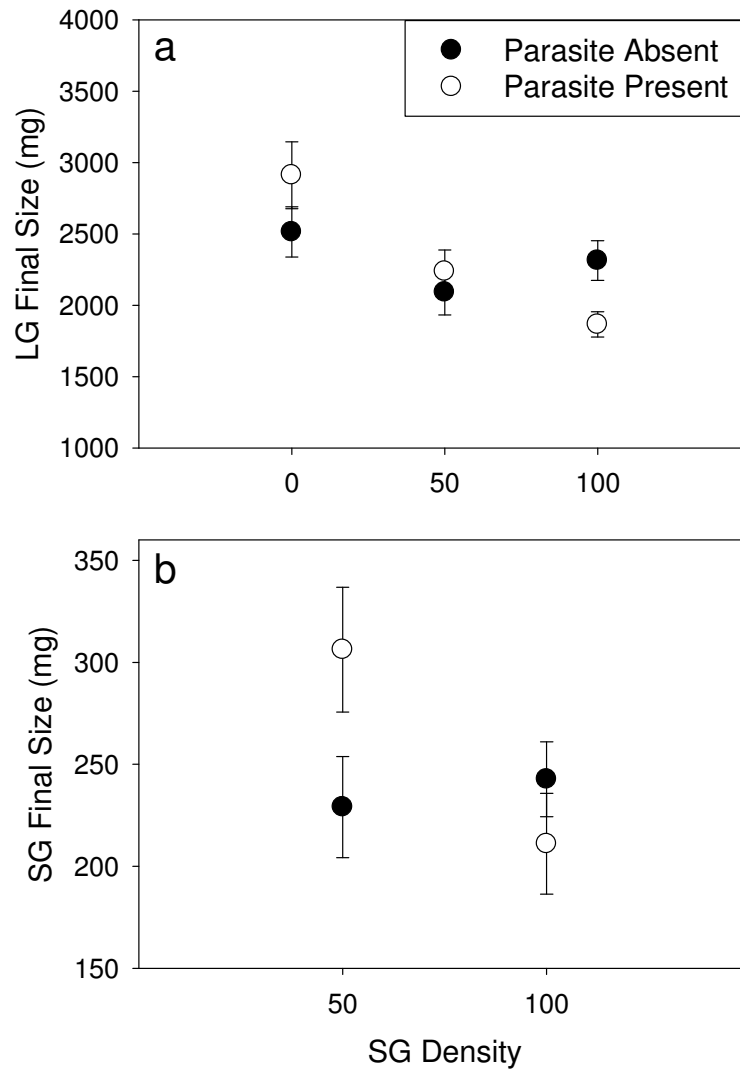
Figure 4.1: a) At higher densities of small green frog tadpoles (SG) in Experiment 1, the presence of parasites (open circles) resulted in decreased final mass of large green frog tadpoles (LG) than in the absence of parasites (solid circles; parasite x density interaction: $p = 0.02$). b) The effects of parasites on SG final mass also depended on density (parasite x density interaction: $p = 0.02$). Parasites had a more positive effect on growth at the 50 SG density. Points show mean ± s.e.m.
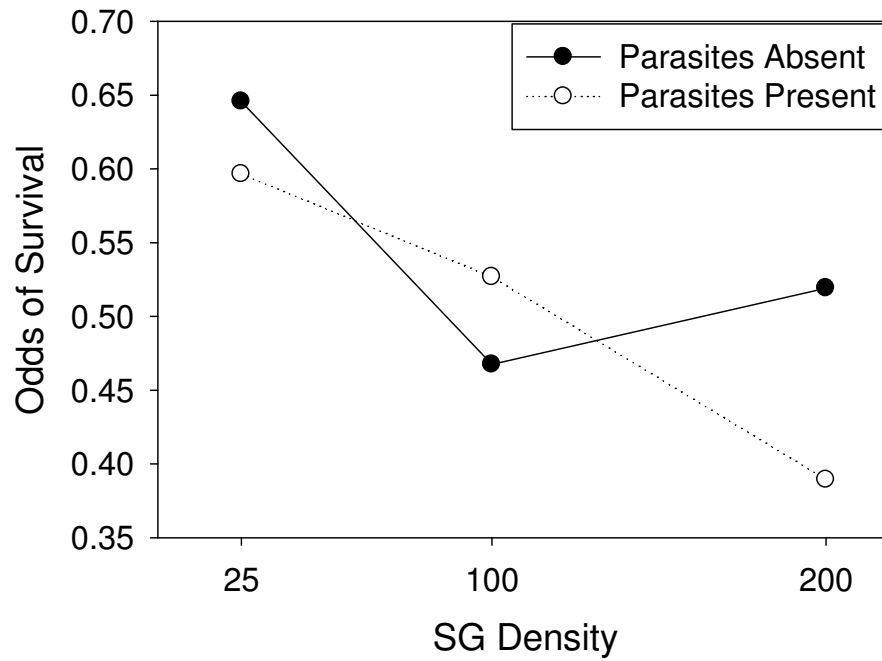
Figure 4.2: Proportional odds of small green frog tadpole (SG) survival depended on the interaction between density and parasite presence in Experiment 2 (p < 0.001). The lowest odds of survival were at the highest density in the presence of parasites. Odds are calculated from the coefficients from the generalized linear mixed effects model (binomial) of survival.
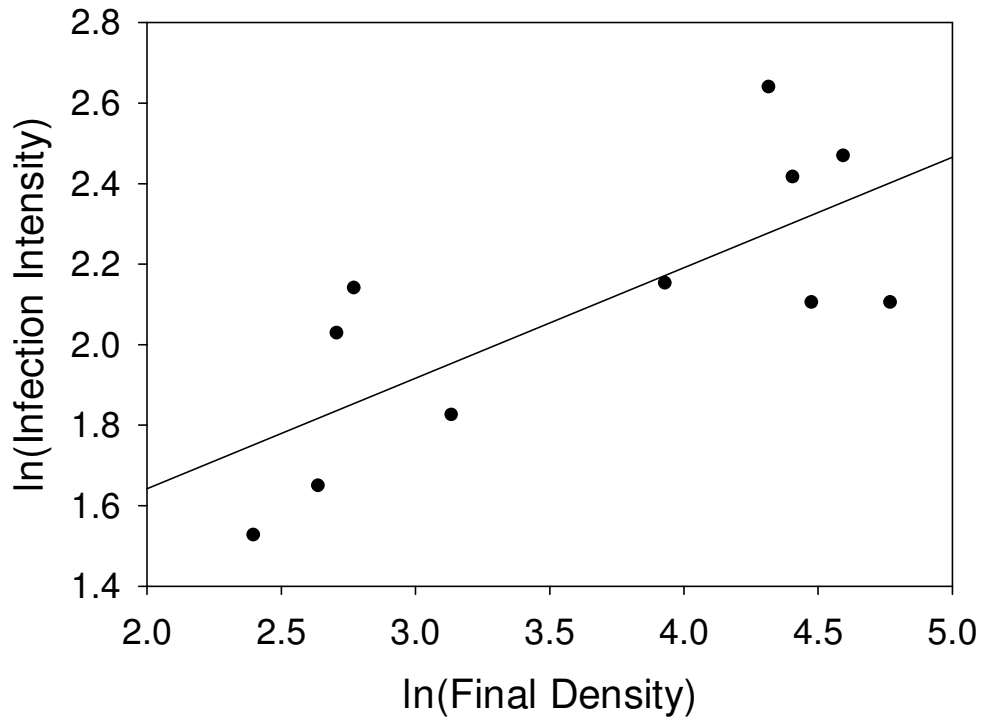
Figure 4.3: Log mean infection intensity (number of metacercariae per individual) of small green frog tadpoles (SG) increased with final SG density (number of surviving small green frog tadpoles) in Experiment 2 (slope = 0.27, R^2 = 0.56, F (1, 9) = 11.29, p = 0.008).
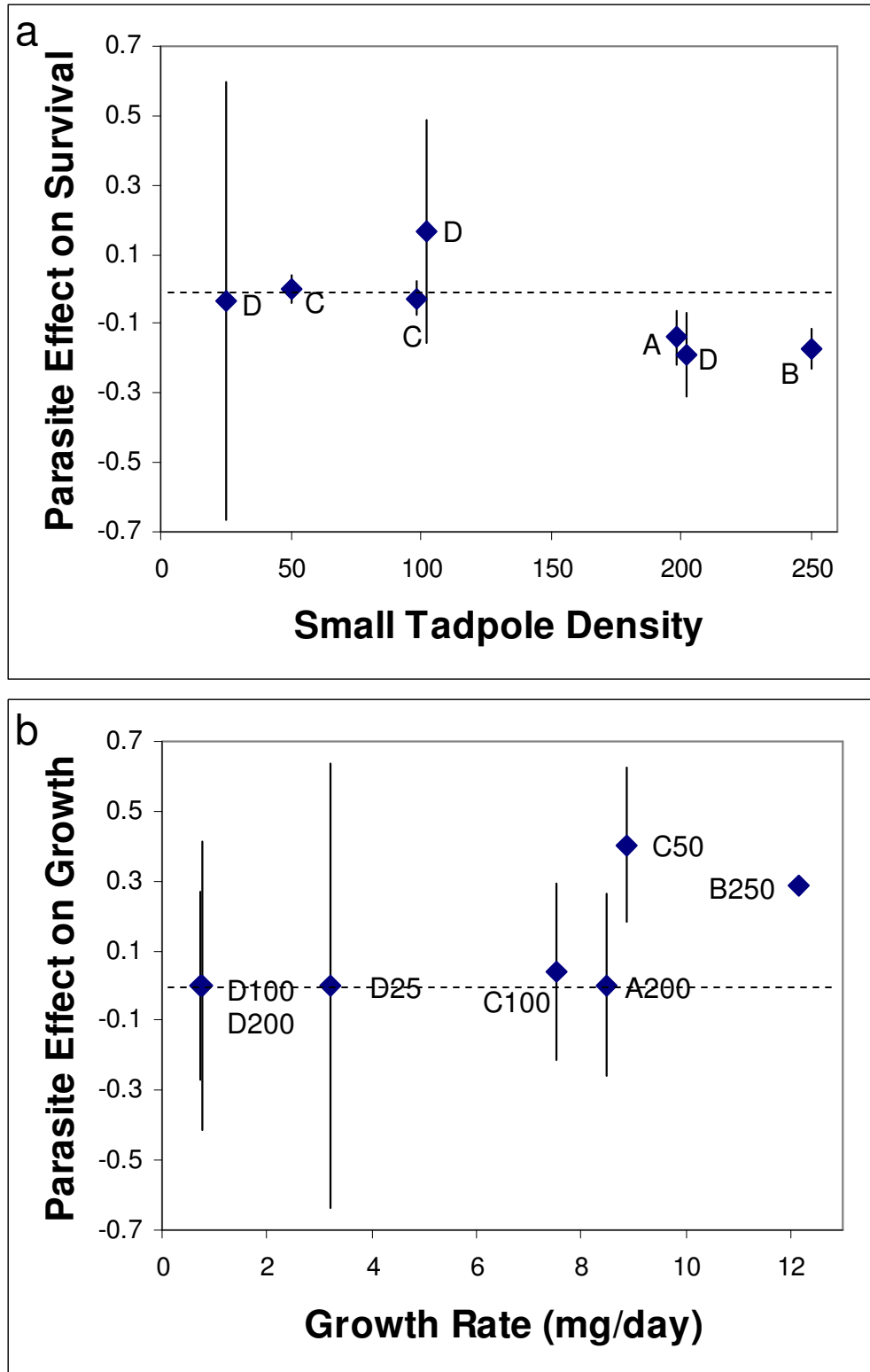
Figure 4.4: a) Effects of parasites on small green frog (SG) growth were more positive at higher absolute growth rates (*p* = 0.02). b) Across four mesocosm experiments, parasites reduced SG survival more at higher densities (*p* = 0.02). Letters indicate experiment (summarized in Table

4.2), and numbers in (a) indicate density. Effect sizes are the log response ratio (parasites/control) for growth rates and survival, calculated for each density within each experiment. Points show mean ± s.e.m.

Appendix: Supplementary methods and results for cross-experiment comparison

To corroborate our findings in our two experiments here, we compared our results with those from two additional experiments that used a similar design. In all four studies (summarized in Table 4.2), mesocosms were set up in the same manner and small green frog tadpoles (initial size ~13 mg, comparable to SG here) were exposed to 3 caged uninfected or infected *P. trivolvis* snails (~1 g). Thus, all experiments used the same exposure level, assuming similar cercariae production from snails. The first additional experiment had an initial density of 200 SG tadpoles (10 removed on day 14), ran for 26 days in 2010, and had 5 replicates. The second additional experiment had an initial density of 250 SG tadpoles (50 removed on day 7), ran for 14 days in 2011, and had 8 replicates. We excluded predator treatment containers in these experiments from analysis to avoid any confounding effect on growth or survival. Only SG were present in the earlier experiments (given that only 5 LG were present in our experiments here, we expect LG had relatively little reciprocal competitive effect on SG). Thus, we were able to assess whether the interactive effects on survival and growth were consistent across all of these experiments. Although these experiments ran for different durations, we expect that most parasite-induced mortality occurred relatively early in experiments, because early-stage tadpoles are most susceptible (Holland et al. 2007). Also, although tadpole growth rates depend on size class (Werner 1986), confounding effects of size on our comparison should be small, given that tadpoles of effectively the same initial mass and stage were used.

For each initial density in each experiment, we calculated log response ratios for growth rates and survival. The relationship between each effect and growth rate was analyzed using mixed effects meta-regression (R metaphor package), including growth rate (mean control) as a moderator variable. The results showed that the magnitude of the growth effect was positively associated with absolute growth rate, but there was not a relationship between growth rate and the survival effect (see Discussion). We note that caution must be employed in comparing results across experiments. The preceding analyses effectively assume that different densities within each experiment can be treated as independent. However, averaging effects within each experiment would likely gloss over potentially important differences among density treatments.

Chapter V

Food resource levels influence larval anuran-echinostome host-parasite interactions

Introduction

Host-parasite interactions can strongly depend on environmental factors that influence host traits (Scrimshaw 2003, Duffy et al. 2011). For instance, intra- and interspecific competition and a range of abiotic factors may influence host food resource availability, which may in turn have positive or negative effects on parasite infection rates and host tolerance of infection (Sandland and Minchella 2003, Bedhomme et al. 2004, Kau et al. 2011, Cornet et al. 2013). Hosts may have elevated growth rates or improved body condition at high resource levels, which may increase resources available to parasites (Hechinger 2013). Higher host resources may thus enhance parasite survival, growth and reproduction, which may overwhelm any positive effect of increased resources on host fitness (Pulkkinen and Ebert 2004, Hall et al. 2009). Alternatively, more and higher quality host resources may facilitate greater host investment in defenses against parasites (Smith et al. 2005, Tschirren and Richner 2006), such as better immune function (Gross and Newberne 1980). The contradictory effects at play create difficulties in predicting potentially important consequences of these effects for parasite transmission and the population-level consequences of parasites (e.g., Pedersen and Greives 2008, Tadiri et al. 2013). More research is thus needed to assess the effects of resource levels on individual host-parasite interactions and the underlying mechanisms, which can be used to generate hypotheses for effects at larger ecological scales.

Effects of resource levels on host-parasite interactions may also be of conservation concern, as human activities often enhance resource levels (Bennett et al. 2001), which can impact parasite abundance and dynamics (Bruno et al. 2003, Johnson et al. 2010, Schotthoefer et al. 2011, Jones et al. 2013, Long et al. 2013). For instance, trematode parasites are positively

associated with eutrophication in aquatic ponds (McKenzie 2007), in part because increased nutrients result in higher densities of snail first intermediate hosts (Johnson and Chase 2004, Johnson et al. 2007, Rohr et al. 2008). However, other effects of eutrophication on trematode parasites are possible that have not been fully explored, such as the consequences of increased food availability for second intermediate hosts (e.g., fish, amphibians), which may amplify or moderate parasite abundances and fitness effects. In particular, parasite effects on amphibians are of concern, given recent global declines in which disease is believed to have played a role (Stuart et al. 2004).

Here, I examined the effects of host food resource levels on interactions between *Echinostoma revolutum* (Digenea: Echinostomatidae) parasites and a common second intermediate host, larval green frogs (*Rana clamitans*). Previous evidence suggests that competition (i.e., increased host densities) and *E. revolutum* parasitism may interactively influence larval frog growth and survival, potentially because higher resource levels at lower densities may allow tadpoles to escape costs of infection (Chapter IV). I thus hypothesized that higher resources would reduce the fitness costs of parasitism, due to increased host investment in parasite defenses. To test this hypothesis, I performed an aquarium experiment in which I exposed larval green frogs to parasites across a gradient of food resource levels and measured behavior, growth, survival, and infection.

Methods

Study System

E. revolutum has a complex life-cycle involving a snail first intermediate host, an amphibian, fish, or mollusk second intermediate host, and a bird definitive host (Kanev et al. 1995, Kanev et al. 2000). Within the snail first intermediate host, the parasite undergoes multiple rounds of asexual reproduction during sporocyst and redia stages before producing infective, free-swimming cercariae. Cercariae leave the snail host and enter the amphibian host through the cloaca and migrate to the kidneys, where they encyst as metacercariae. Echinostomes have a range of effects on amphibian hosts, such impaired kidney function and death (Holland et al. 2007), reduced growth rate (Marino et al., in press, Fried et al. 1997), an induced behavioral avoidance response to cercariae (Koprivnikar et al. 2006, Rohr et al. 2009), and reduced activity levels post-infection (Thiemann and Wassersug 2000, Marino et al., in press). Green frog larvae

are common hosts (Skelly et al. 2006, Chapter VII), and larger tadpoles at later developmental stages are less affected by infection (Holland et al. 2007).

Animal collection and care

Green frog egg masses were collected from the Edwin S. George Reserve (ESGR) experimental ponds and placed in 300 L wading pools filled with aged well water. After hatching, tadpoles were fed Purina® Rabbit chow ad libitum until the beginning of experiments. During experiments, tadpoles were fed with 3:1 Purina® Rabbit Chow: Tetramin® fish flakes mixture three times per week, and water changes were performed weekly. Water in the laboratory was reverse osmosis, UV-filtered well water to which 63 mg/L of API aquarium salt was added. Aquaria (8L; 26 x 38 x 14 cm) were maintained under full-spectrum lighting at 14:10 h light: dark cycles. Research using these animals was performed in accordance with University of Michigan UCUCA Protocol #07765.

To obtain parasites, *Planorbella trivolvis* snails were collected from three ponds in Livingston County, MI (ponds referred to as Duck Pond [42.481308, -83.983442], Kaiser South Pond [42.430299, -84.036582], and East Marsh [42.45679, -83.996748] in Marino et al., in press). Echinostomes in snails from these ponds were previously identified as *Echinostoma revolutum* using molecular methods (Marino et al., in press). Snails were screened for trematode infection by placing them in 60 mL water in cups under a 60 W light. After 4 h, all cups were examined for the presence of trematode cercariae under a dissecting microscope. A few cercariae were then placed in 70% ethanol and identified as echinostomes by morphology using a taxonomic key (Schell 1985).

Experimental Design

To examine the influence of food resource levels on parasite susceptibility, I performed a 2 x 2 factorial aquaria experiment in which tadpoles were placed in aquaria with an infected or uninfected *P. trivolvis* snail and fed either high or low food levels. I used a randomized block design with eight replicates. On July 10, 2012, 10 tadpoles (28.7 ± 1.8 mg, from 4 egg masses collected June 11) were moved into each aquarium. Tadpoles in the high food treatment were fed 12% of their body mass for the duration of the experiment, and tadpoles in the low food treatment were fed 3% of their body mass for the first two weeks of the experiment. I then reduced food levels in the low food treatment to 1% for the final week of the experiment after I did not observe any interactive effect of food level and parasite exposure on growth or survival

during the first two weeks, in order to test if a more extreme food reduction would have an effect. Tadpoles were fed three times per week, and water was changed weekly. On Day 3, I performed one set of 10 behavior observations over two hours. During each observation, the observer slowly approached each aquarium and counted the number of active (moving) tadpoles over a 5 s interval. I also counted the number of tadpoles at the surface of the aquaria during each observation to measure a potential behavioral response of tadpoles to cercariae. In order to account for variation in cercariae production by individual snails, infected and uninfected snails were rotated within each block between high and low food level treatments after each feeding. I replaced any snails that died during the experiment. Tadpoles were weighed weekly, and the experiment was terminated after three weeks. All tadpoles were euthanized and preserved, and I later dissected five tadpoles from each container, or all tadpoles when fewer. I removed and dissected the kidneys (mesonephri and pronephri) and nephric ducts and counted the number of metacercariae.

Statistical analyses

All analyses were performed in the R statistical package v.2.15 (http://www.r-project.org/). Log-transformed infection intensity (total number of metacercariae) and final mass and arcsine-square root transformed activity levels and water column location were analyzed using ANOVA. Survival was analyzed using generalized linear mixed effects models (GLMM) with a binomial distribution and block as a random effect, using the glmer function in the R lme4 package. I also performed additional analyses on the parasite treatment data to further elucidate the relationships between tadpole size and infection intensity using linear models and between infection scaled to mass and survival using GLMM. For the latter, I analyzed the relationship of infection scaled to host mass (i.e., mean number of metacercariae per mg of host in surviving tadpoles) with survival (number of individuals surviving per tank), with food level and the food level x scaled infection interaction as covariates. Using mass as a scaling factor should at least partly correct for size-based differences in infection susceptibility (Holland et al. 2007). I assumed that variation in metacercariae per unit mass was caused by natural variation in cercariae production among snails and could be treated as an independent variable, although I note some caveats of this assumption in the Discussion.

Results

One "uninfected" snail had a latent infection and caused infection in tadpoles in one block, so the two affected containers were excluded from analysis. Final mass was greater at the higher food level (Figure 5.1a; $F (1, 18) = 243.22$, $p < 0.001$) but did not depend on the presence of parasites ($F (1, 18) = 0.76$, $p = 0.8$), although the parasite x food level interaction was marginally non-significant ($F (1, 18) = 4.05$, $p = 0.06$). With respect to infection, metacercariae generally occurred in the kidney tissue, although a single tadpole in a container at the 3% food level had 415 metacercariae present in its intestine. Whether or not that anomalous individual was included in analysis, infection intensity was significantly greater at the higher food level (Figure 5.1b; outlier excluded: $F (1, 7) = 62.21$, $p < 0.001$; outlier included: $F (1, 7) = 20.23$, $p = 0.003$). Activity levels did not depend on resource levels ($F (1, 19) = 0.41$, $p = 0.5$), parasite exposure ($F (1, 19) = 0.12$, $p = 0.7$), or the food level x parasite interaction ($F (1, 19) = 2.41$, $p = 0.14$). The proportion of tadpoles at the top of the water column was greater in the aquaria that contained infected snails (Figure 5.1c, $F (1, 19) = 32.62$, $p < 0.001$), but did not depend on food levels ($F (1, 19) = 0.60$, $p = 0.4$) or the food level x parasite interaction ($F (1, 19) = 0.45$, $p = 0.5$). Survival decreased in the presence of infected snails (Figure 5.1d, $z = -2.337$, $p = 0.02$), but did not depend on food level ($z = -0.47$, $p = 0.6$) or the food x parasite interaction ($z = 0.98$, $p = 0.33$).

The relationship between size and infection intensity was positive and nonlinear, with a polynomial model significantly better than a linear model (Figure 5.2a; $F (1, 13) = 13.8$, $p = 0.003$) and also a better fit than a logarithmic model ($\Delta AIC = 6.56$). In the analysis of survival using mass-scaled infection, survival decreased with scaled infection ($z = -2.46$, $p = 0.013$), did not depend on food levels ($z = -1.411$, $p = 0.16$), and the interaction between scaled infection and food level was significant (Figure 5.2b; $z = 2.02$, $p = 0.043$). At low food levels, survival decreased more rapidly with increased infection intensity compared to at high levels.

Discussion

The results demonstrate that resource levels can mediate host-parasite interactions. Under continuous exposure to echinostome cercariae, higher infection intensities occurred in green frog tadpoles fed higher resource levels than those fed lower resource levels. As metacercariae do not actively reproduce within amphibian hosts, the most likely explanation for this effect was differential infection rates depending on host size, which has been documented elsewhere

(Holland et al. 2007, Chapter IV). Tadpoles grew more than twice as much at higher food levels than at lower food levels and experienced a corresponding increase in infection intensity.

Several factors may contribute to differences between host sizes in infection. Larger hosts may experience higher infection because they are easier to locate or may exhibit weaker physiological or behavioral responses to parasites. In addition, the increase in total infections with resource levels is consistent with a hypothesis that intraspecific competition occurs among cercariae, which may be alleviated by an increase in available host tissue in larger hosts. A possible alternative is that, despite rotations of snails among tanks, more cercariae were released by snails in the higher resource treatments. However, as larger tadpoles have been shown to experience higher infection, both when co-occurring with smaller hosts (Chapter IV) or when different size classes are exposed separately (Holland et al. 2007), tadpole size-dependent infection rates are the likeliest explanation. The consequences of increased infection intensities at higher resource levels are both more infections per host as well as more total infections, with potential implications for host-parasite dynamics.

My findings also reveal further subtleties in the relationship between parasite infection and host size. The nonlinear relationship between host final mass and infection suggests that, at small sizes, host size restricts infection intensity (i.e., due to factors discussed above), while other factors limit infection when hosts are larger. The saturating portion of the curve is consistent with a limited number of cercariae produced per snail host. At larger sizes, infection may be limited by the number of cercariae produced rather than host size. The leveling-off or slight decline on right portion of the hump-shaped curve, as suggested by the polynomial fit, could also be driven in part by higher rates of cyst elimination at larger sizes (Holland 2009). Further research explicitly controlling for individual size and exposure levels could provide additional insights into how the balance between new infections and cyst elimination influences the relationship between size and infection.

The relationship between host size, infection intensity, and fitness effects of parasites likely depends on several balancing factors. Increased infection intensity due to higher growth rates could be expected to increase fitness costs of infection, due to intensity-dependent effects (Marino et al., in press). However, a counteracting effect of increased growth on fitness also occurs, because with increased size due to faster growth enhances tolerance of infection (Holland et al. 2007), which would be expected to decrease fitness costs of infection. Depending on the

strength of these counteracting effects and nonlinearities in the functional relationship, increased growth caused by higher resource levels could amplify or nullify the effects of parasites on infection.

To this question, the results provide some support for my hypothesis that high resource levels reduce the fitness costs of parasitism. Although an interaction did not occur between parasite presence/absence and food level, an interaction between scaled infection intensity and food level occurred in the parasite treatment. The latter interaction suggests that increased infection may be more costly at higher food levels, which is generally consistent with a negative interactive effect of echinostome parasitism and competition (Koprivnikar et al. 2008, Chapter IV). A caveat of this interpretation is that infection per mg host biomass was not explicitly controlled, and thus the pattern may not purely represent a causative relationship. Nonlinearities in the relationship between host mass and infection and the relationship between host number per container and infection rates (e.g., due to an increased ratio of cercariae to hosts) could potentially influence the observed relationship between scaled infection and survival.

Surprisingly, the parasite treatment did not significantly affect growth, in contrast to a previous study using a one-time exposure to cercariae (e.g., Chapter II), although we did observe a marginally non-significant interactive effect of resource levels and parasitism. This marginal interaction results from a trend that tadpoles actually grew more in the presence of parasites at the low food level. However, these higher growth rates may have resulted from cannibalistic necrophagy, rather than any positive effect of parasites on tadpole growth per se, because I repeatedly observed live tadpoles consuming dead tadpoles before I was able to collect them. Necrophagy may have especially enhanced growth rates in the low food-parasite treatment where food was limited and high mortality occurred.

An additional, novel result here is that tadpoles exhibited vertical migration behavior in response to parasite presence. The behavioral response is likely avoidance behavior in response to cercariae, rather than parasite manipulation, as such an effect was not observed previously in experiments using a one-time rather than continuous exposure to parasites (e.g., Chapter II; Marino, pers. obs.). Such avoidance behavior is likely adaptive, as *E. revolutum* cercariae move downward in the water column (Loy et al. 2001). Vertical avoidance may complement avoidance behaviors such as increased activity (Koprivnikar et al. 2006, Daly and Johnson 2011) or horizontal spatial avoidance (Rohr et al. 2009) in response to trematode cercariae. A behavioral

response may have important consequences for other ecological interactions. For instance, vertical migration may contribute to increased visibility and susceptibility to predators, which may contribute to a synergistic effect of echinostomes and predators on larval frog survivorship (Marino and Werner, in press).

The effects of resource levels shown here are likely relevant in wild populations and may have important consequences for host-parasite population dynamics, as observed infection intensities are well within the range observed in larval green frogs in ponds (Skelly et al. 2006, Chapter VII). Increased infection establishment by cercariae may amplify other positive effects of increased resources on parasite populations across life stages, such as increased snail abundance, with potentially dire consequences for host populations at larger scales. These results thus also have implications for amphibian conservation, as human activities (e.g., non-point source pollution) are causing increased productivity in freshwater bodies (Bennett et al. 2001). Eutrophication may have a range of simultaneous effects on amphibians and parasites, from the individual to ecosystem scale. Understanding how such factors influence parasite transmission and the overall effects on hosts will be crucial to appreciate the consequences of human actions for affected populations.

Acknowledgements

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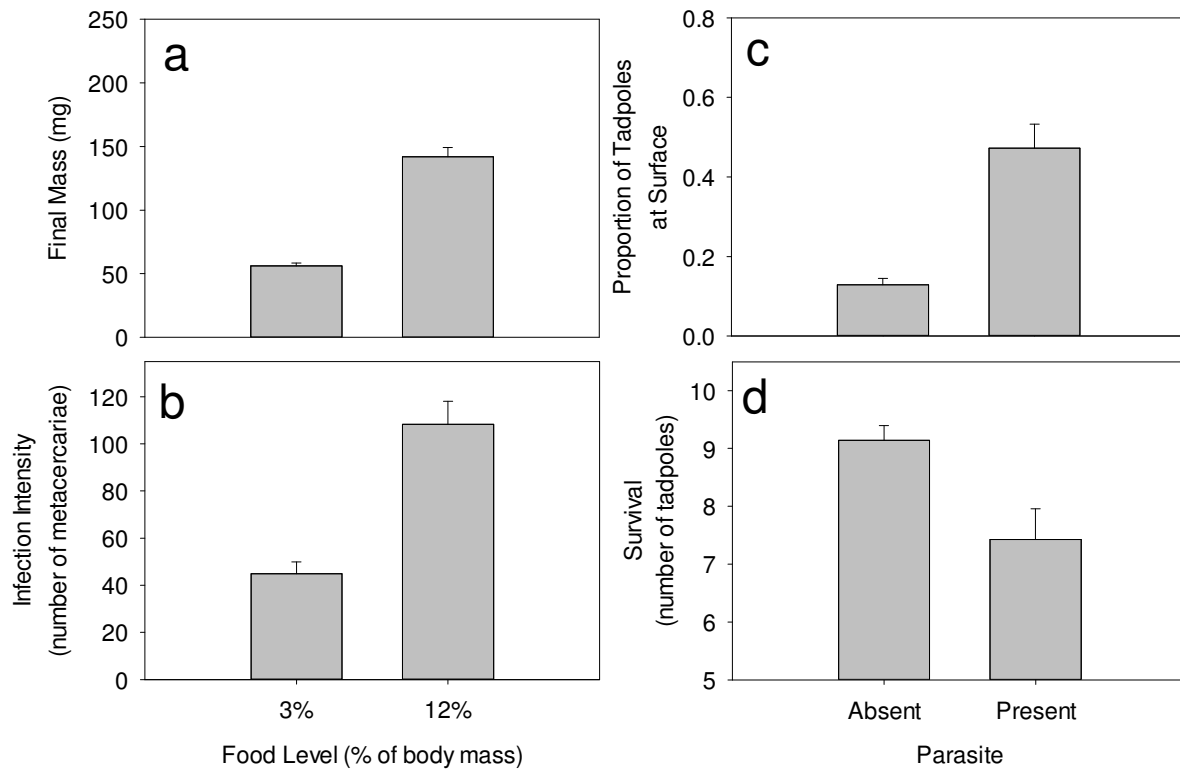
Figure 5.1: a) Final mass of tadpoles was greater at higher food levels ($p < 0.001$). b) Mean final infection intensities were greater at higher food levels ($p < 0.001$). c) A larger proportion of tadpoles occurred at the surface of aquaria ($p < 0.001$). d) The presence of parasites reduced the final number of surviving tadpoles ($p = 0.02$). Tadpoles in groups of 10 were placed in 8 L aquaria with either one uninfected or infected snail and fed either high or low food levels for three weeks.
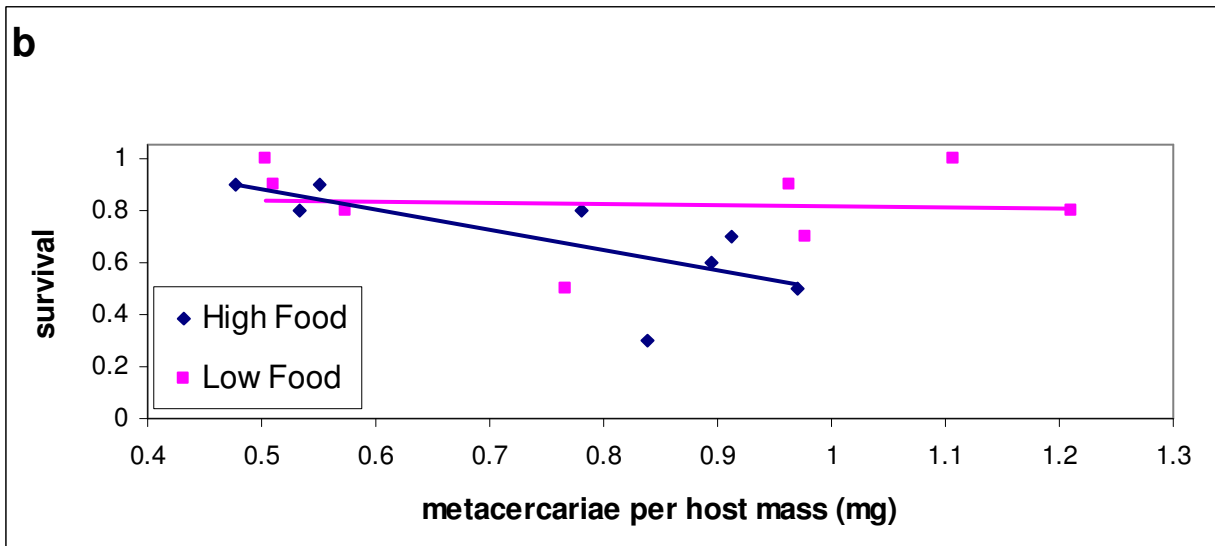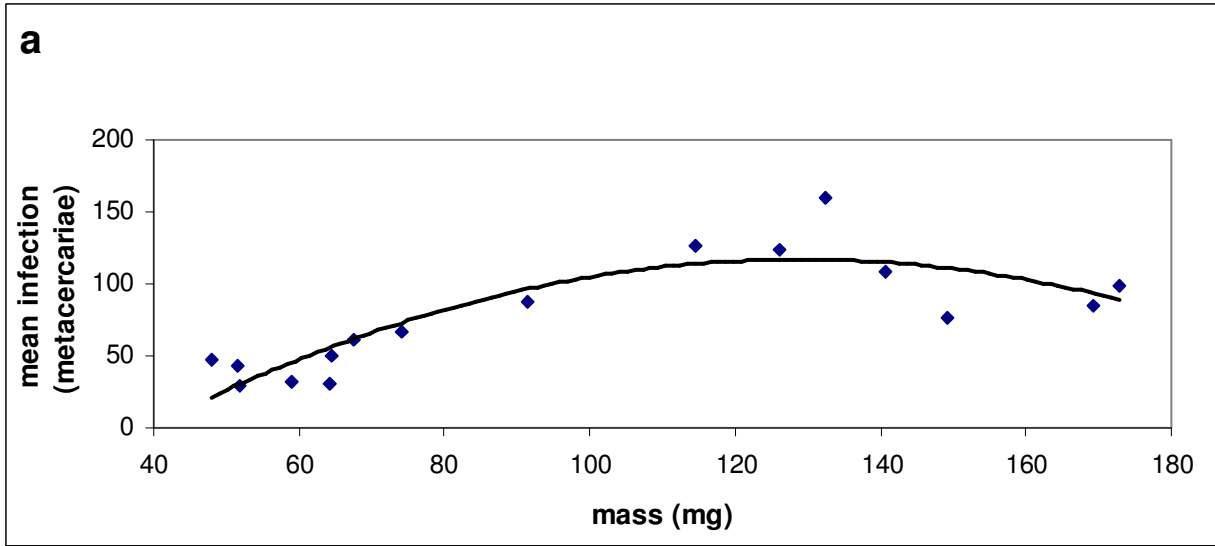
Figure 5.2: a) Infection intensity increased nonlinearly with host mass across treatments exposed to parasites ($R^2 = 0.77$, p = 0.003). b) Survival decreased with the number of parasites per host biomass at low food levels more than at high food levels (infection x food interaction: $p = 0.04$). Trend lines added to show overall direction of effects.

Chapter VI

Susceptibility of eight anuran species to trematode parasites across ecological contexts

Introduction

Understanding the relationship between biodiversity and disease is of vital importance for predicting the emergence of infectious diseases (Daszak et al. 2000, Jones et al. 2008) and integrating parasites into food webs (Lafferty et al. 2006, Lafferty et al. 2008). A crucial step in developing this understanding will be to assess differential effects of parasites on co-occurring host species. For example, differential effects of generalist pathogens on host species can affect disease dynamics through dilution, amplification, or decoy effects (LoGiudice et al. 2003, Begon 2008, Johnson and Thieltges 2010) and community structure through apparent competition (Hudson and Greenman 1998, Tompkins et al. 2000) and keystone effects (Hatcher et al. 2006, Patot et al. 2012). Such effects may occur due to differences in three components of susceptibility: avoidance (behavioral reduction in exposure to parasites), resistance (physiological reduction in infection loads), and tolerance (ability of hosts to minimize fitness costs associated with a particular infection load) (Medzhitov et al. 2012). These components of susceptibility are not necessarily fixed species attributes, but can be affected by community context (Hall et al. 2005). For instance, nonconsumptive effects of predators can strongly affect prey traits (Boonstra et al. 1998, Scheuerlein 2001, Middlemis Maher et al. 2013), which may impair the ability of hosts to avoid, resist, or tolerate parasite infection (Thiemann and Wassersug 2000, Ramirez and Snyder 2009, Duffy et al. 2011). It is therefore essential to compare species' susceptibility to parasites across different contexts (e.g., in the presence or absence of predators) in order to predict disease emergence or food web interactions.

Differences in species' susceptibility may also provide valuable insights into the evolution of natural enemy interactions. For instance, species more strongly associated with

parasites due to habitat use may evolve greater resistance to infection, and habitat-driven variability in species' exposure to parasites may lead to the evolution of plasticity in avoidance behavior (Hart 1990). Consequences of habitat use for susceptibility may interact with other factors that also can influence susceptibility to parasitism, such as life history (Arriero and Moller 2008, Johnson et al. 2012) and breeding phenology (Calero-Torralbo et al. 2013). Furthermore, community context can also influence the evolution of host-parasite interactions (Duffy et al. 2012), e.g., predators can have strong effects on prey traits (e.g., behavior, Anholt et al. 2000, Relyea 2001a) that influence parasite susceptibility and cause interactive effects (Thiemann and Wassersug 2000, Ramirez and Snyder 2009). Species may thus adapt in response to interactive effects of predators and parasites rather than each enemy independently. Such interactive effects may differ among species because of differential responses of species to tradeoffs in susceptibility to parasites and predators (e.g., Marino and Werner, in press, Marino et al., in press). Comparative studies of species may provide key insights into these tradeoffs.

Larval anurans provide an ideal system for comparative studies of the effects of natural enemies (e.g., Relyea 2001a, Rohr et al. 2010). Here, I tested two hypotheses that address how species traits and community context influence anuran susceptibility to parasites. First, I hypothesized that species differ in susceptibility to parasites due to trait differences (i.e., habitat use, life history, and phenology). Second, I hypothesized that predator presence differentially affects the susceptibility of species to parasites, due to differential responses of species to tradeoffs between susceptibility to each natural enemy. For example, behavioral responses to parasites may enhance susceptibility to predators (Thiemann and Wassersug 2000, Szuroczki and Richardson 2012), or vice versa (Belden and Wojdak 2011, Chapter III). As a result, species differences in associations with these natural enemies may influence how they respond to parasites, predators, and the combination, with potential consequences for parasite transmission. To test these hypotheses, I compared the responses of eight species of larval anurans from three families (Ranidae, Hylidae, and Bufonidae) to trematode parasites (Digenea: Echinostomatidae), predator presence, and the combination of parasites and predators. To determine the mechanisms responsible for species differences, I then assessed how different components (avoidance, resistance, and tolerance) contribute to overall susceptibility, and I evaluated the relationship between susceptibility and host traits. Finally, to determine the importance of community

context, I evaluated how differences among species in susceptibility depended on predator presence.

Study system

Echinostomes have a complex life cycle involving three hosts: a mollusk first intermediate host, an amphibian, fish or mollusk second intermediate host, and a vertebrate definitive host (Najarian 1953, Kanev et al. 2000). Free-living infective stages, cercariae, leave a snail host and enter into a larval amphibian host via the cloaca, after which the parasites encyst in the kidney as metacercariae. Echinostomes can affect growth (Fried et al. 1997) and survival of amphibian hosts at high infection levels, especially when hosts are at early developmental stages (Holland et al. 2007). Anuran species vary in their susceptibility and phenotypic responses to both echinostomes (Holland 2010, Rohr et al. 2010) and predators (Van Buskirk 2000, Relyea 2001b, Relyea 2001a), and the combination of these natural enemies may have interactive effects (Thiemann and Wassersug 2000, Szuroczki and Richardson 2012).

I compared the separate and joint effects of echinostomes and predators on eight species that differ in development rate, habitat use, and phenology, all of which may affect their susceptibility to parasites and predators. Spring-breeding species included wood frogs (*Rana sylvatica*), northern leopard frogs (*Rana pipiens*), spring peepers (*Pseudacris crucifer*), western chorus frogs (*Pseudacris triseriata*), and American toads (*Bufo americanus*). Summer breeding species included gray tree frogs (*Hyla versicolor*), bullfrogs (*Rana catesbeiana*), and green frogs (*Rana clamitans*). Echinostome prevalence in snail hosts increases throughout the summer (Sapp and Esch 1994), so that summer-breeding amphibians tend to experience greater exposure to parasitism than spring-breeders. In addition, infection intensities in amphibians correlate positively with snail densities (Skelly et al. 2006, Rohr et al. 2008), so that amphibian species that inhabit ponds more suitable to snail hosts also experience greater exposure to parasitism. Finally, development rates may influence susceptibility, as faster-developing species spend less time in the aquatic larval stage, generally leading to a shorter periods of exposure to trematode cercariae, which would reduce selection for parasite defenses. A consequence is that susceptibility to trematode parasites tends to increase with "pace of life" (Johnson et al. 2012).

Methods

Two experiments were conducted in aquaria (8 L, dimensions: 26 x 38 x 14 cm) in order to examine different components of susceptibility in the absence or presence of predators. The first experiment focused on initial infection and parasite avoidance behavior, and the second experiment focused on post-parasite exposure effects on survival and traits.

Animal collection and rearing

Eggs or breeding pairs of each species were collected on or near the Edwin S. George Reserve (ESGR) in Livingston County, MI, during spring and summer 2011. Eight sets of chorus frog egg masses were collected from Buffer Zone Marsh. Chorus frog females lay sets of multiple small egg masses near a breeding male (Whitaker 1971), so sets were collected at least 5 m apart from each other to ensure that they were from different males. Eggs were collected from 12 breeding pairs of spring peepers collected from three natural ponds on the ESGR. To collect the eggs, individual female spring peepers were placed with three males in 1 gallon buckets floating in ponds or 11 L aquaria (containing ~5 cm pond water, oak leaves, and twigs) in the laboratory. Ten wood frog egg masses were collected from Southeast Marsh, 5 leopard frog egg masses from a pond two miles northeast of the ESGR (hereafter Duck Pond), 10 American toad egg masses from a small pond 100 m east of the ESGR, and 7 green frog egg masses and 2 bullfrog egg masses from the ESGR experimental ponds. Eggs and hatchlings from 7 gray tree frog pairs were collected from open 300 L plastic pools (deliberately set up to provide breeding habitat) or from pools of rainwater that formed in overturned cattle watering tanks.

Eggs and hatchlings were moved to 300 L wading pools filled with aged well water and covered with 60% shade cloth. After hatching, tadpoles were fed Purina® rabbit chow ad libitum until reaching an average mass ~22 mg (Table 6.1), at which point they were used in experiments. Tadpoles were of similar size for all experiments to control for size-dependent differences in susceptibility (Schotthoefer et al. 2003, Holland et al. 2007). Due to differences in breeding time and development rates, experiments were necessarily initiated on different dates for each species. Water used in the laboratory was reverse osmosis, UV-filtered with added 63 mg/L API aquarium salt. Tadpoles were fed 6% of their biomass per day with 3:1 Purina® Rabbit Chow: Tetramin® fish flakes mixture. All aquaria were maintained under full-spectrum lighting at 14 h light: 10 h dark cycles, and temperature was maintained between 20-22 °C.

Parasites used in experiments were from *Planorbella trivolvis* snails collected from Duck Pond, East Marsh, and a pond located 2.5 miles southeast of the ESGR (hereafter, Kaiser South

Pond). Previous analysis of a 28S ribosomal DNA gene from echinostomes in *P. trivolvis* from these ponds indicated that this species was likely *Echinostoma revolutum* (Marino and Werner, in press). To collect cercariae, snails were placed under 60 W lights in cups of 60 mL water, and cercariae were transferred to a Petri dish with a pipette. Cercariae were then counted and moved into 60 mL of water in cups following the methods of Holland et al. (2007). Cercariae were introduced to experimental animals within 8 h of leaving the snail host. Predators were a mixture of late-instar larval *Anax junius* and *A. longipes*, common predators of larval frogs. This research was performed in accordance with University of Michigan UCUCA Protocol #07765.

Experiment I: Effects of predators on parasite avoidance behavior and initial infection

The first experiment examined parasite avoidance behavior and infection establishment in the absence or presence of predator cue, using a randomized block design with 10 replicates. Five tadpoles were moved into each aquarium, fed, and allowed at least 2 h to acclimate. To generate predator cue, 10 *Anax* predators in 1 L deli cups were fed ~100 mg tadpoles, and the water from those cups was then mixed to homogenize the cue and divided equally among appropriate aquaria, after Fraker (2008). The equivalent volume of water was added to control aquaria. 30 min after predator cue addition, 10 sets of behavior observations were performed over 30 min. During each observation, the observer slowly approached each aquarium and counted the number of tadpoles that were active during 5 s intervals. After behavior observations were completed (1 h after predator cue addition), 200 cercariae were added to each aquarium. After tadpoles had been exposed to cercariae for 15 min, an additional 10 sets of behavior observations were performed over 30 min. After 48 h, all tadpoles were euthanized and preserved in 70% ethanol for later dissection. The mesonephri, pronephri, and nephric ducts of each tadpole were dissected and the number of echinostome cysts counted. Log-transformed infection levels (number of metacercariae) were analyzed using a linear mixed effects model, with block as a random effect. Activity levels were analyzed using a linear mixed effects model with repeated measures, with block and aquarium as random effects. These and all subsequent analyses were performed in the R statistical package v.2.15 (http://www.r-project.org/).

Experiment II: Effects of predators on post-infection survival and traits

Experiment II examined post-parasite exposure survival and traits in the presence or absence of predators, removed from any effect of predators on initial infection establishment (e.g., Thiemann and Wassersug 2000), which was the focus of Experiment I. In contrast to

Experiment I, in which tadpoles were simultaneously exposed to predator cue and cercariae, tadpoles in this experiment were exposed to parasites prior to exposure to predator cue. The experiment used a 2 x 2 factorial design in which tadpoles were exposed to 0 or 25 echinostome cercariae and then exposed to caged predators or empty cages. Tadpoles were moved from outdoor pools into the laboratory and allowed at least one hour to acclimate. Individual tadpoles were then placed in 60 mL cups containing 0 or 25 cercariae and left overnight (12-18 h). After the exposure period, 10 exposed and 10 unexposed tadpoles from cups were euthanized and preserved for later dissection and staging (Gosner 1960) to estimate initial infection intensity and developmental stage of each species. Five tadpoles were then moved from the remaining cups into each aquarium, with eight replicates (32 aquaria) for each species. To manipulate the presence or absence of predator cue, aquaria contained an *Anax* predator in a cage (10 x 10 cm slotted drain pipe enclosed with window screening by rubber bands) or an empty cage. Predators were fed ~100 mg conspecific tadpoles three times per week. For chorus frogs and leopard frogs, congeneric tadpoles (spring peepers and wood frogs, respectively) were used to feed predators for the final 1 week and 3 weeks of the experiment, respectively, because conspecific tadpoles were unavailable (tadpoles respond similarly to cue from predators feeding on conspecifics and congenerics, Relyea and Werner 2000). Tadpoles were fed three times per week and water changed weekly. Behavior observations (10 sets over 2 h) were performed 4 and 8 days following the initial exposure to parasites, and tadpoles were weighed at two and four weeks. After four weeks, surviving tadpoles were euthanized, preserved in 10% buffered formalin, and staged (Gosner 1960).

The effects of parasite infection, predator presence, and species identity on survival were analyzed using a binomial generalized linear mixed effect model. Eight models were constructed that included all possible combinations of interactions between the effects of predator presence, parasite infection, and species identity, and the best-fit model was determined using Akaike Information Criteria (AIC). Initial infection intensity and tank mean final mass, activity levels (proportion of tadpoles active), and development rate (calculated as final - initial Gosner stage) were analyzed using ANOVA. Infection intensity and mass were log transformed, development rate was log (x + 1) transformed, and activity level was arcsine square root transformed for analysis.

Relationships among components of susceptibility across species

To provide insight into the contributions of different components to overall susceptibility, across-species correlations among avoidance behavior, infection levels, and post-infection survival were tested using Pearson correlation tests. A strong correlation between avoidance and infection levels would suggest that behavior may contribute to differences among species in infection rates. Weak or no correlation between infection levels and post-infection survival would suggest that differential tolerance or post-infection resistance (rather than only differences in initial infection establishment) contributes strongly to differential susceptibility. Finally, a negative correlation between avoidance behavior and post-infection survival may suggest that hosts which are most threatened by parasites respond most strongly behaviorally. Avoidance behavior for each species was calculated as the mean proportional change in activity after parasite addition in the absence of predator cue in Experiment I. Because the focal response variable in Experiment I was infection levels, there were no controls that received no parasites, but a reasonable assumption is that the difference in activity before and after parasite addition is due to the documented avoidance response of tadpoles to trematode cercariae (Rohr et al. 2009, Daly and Johnson 2011). Infection levels (log-transformed number of metacercariae) from both experiments were used in separate analyses, because host-parasite contract rates in groups at the aquarium scale may differ from individual infections in cups, due to behavioral interactions among tadpoles in aquaria and the different spatial scales used. Finally, survival post-infection was calculated as the proportional decrease in survival after parasite exposure relative to controls (Experiment II).

Relationship between susceptibility and traits across species

The relationship between susceptibility and other traits (development rate and habitat use) was tested using linear models. Development rate was calculated as species mean development rate (final – initial Gosner stage) in control treatments in Experiment II. Species' rank development rate using this measure is consistent with previous studies (Skelly 1995, Relyea 2001a) and thus reasonably representative. For habitat use, the analysis focused on species' habitat overlap with snail hosts. Overlap was determined from 2007-2010 survey data from a biannual (May and July) survey of 37 ponds on the ESGR (Werner et al. 2007a, Hoverman et al. 2011). Echinostome infection occurred at least once in 21 of the 22 surveyed ponds where snails are present on the ESGR over this period (J. Marino and M. Holland, unpublished data). May or July snail densities were used for spring or summer breeding species,

respectively, to correspond to when each amphibian species occurs in ponds at a comparable developmental stage to experiments. For the subset of ponds in which each species occurred across years, I calculated the mean and standard deviation of the combined density (catch per unit effort, from timed dipnet sampling) of three species of snails that are common hosts of echinostomes (*Planorbella trivolvis*, *Stagnicola elodes*, and *Physa gyrina*). Given the strong positive relationship between snail densities and echinostome infection levels (Skelly et al. 2006, Rohr et al. 2008), the calculated mean and standard deviation provide approximations of the risk of infection that each species experiences and the variation in that risk over space and time, respectively. A relationship between habitat variation and avoidance behavior is especially likely, because avoidance response is a plastic trait, which thus may be favored by more variable environments (Thompson 1991, Van Buskirk 2002). Initial models for each component of susceptibility thus contained three potential predictors: development rate and associated snail density mean and variation, which were log(+1) or log-transformed prior to inclusion in analyses. Final models were selected using AIC.

In order to control for the potential influence of phylogenetic inertia, phylogenetic independent contrasts (PICs, Felsenstein 1985) were calculated for each trait and component of susceptibility using the pic function in the ape package in R (Paradis et al. 2004). A phylogenetic tree was created using well-supported relationships among taxa (Figure 6.1, Bossuyt et al. 2006, Frost et al. 2006, Hillis and Wilcox 2006). Divergence times estimated by Bossuyt et al. (2006) were used within the ranids and from Timetree (Hedges et al. 2006) for the other relationships. For two pairs of congeners for which divergence times were unavailable (i.e., between green frogs and bullfrogs and between chorus frogs and spring peepers), branch lengths were divided equally among taxa. I also tested for phylogenetic signal by calculating the K statistic (Blomberg et al. 2003), using the phylosignal function in the picante package in R (Kembel et al. 2010).

Finally, I also tested for correlations between each component of parasite susceptibility and species' behavioral responses to predators (proportional change in activity in response to predator presence relative to controls in Experiment II). Species which show low parasite susceptibility may be more susceptible to predators due to potential tradeoffs in susceptibility (e.g., Marino and Werner, in press). Thus, a correlation between parasite susceptibility and the behavioral response to predators may be likely, because the behavioral response to predators can be correlated with predation risk (Relyea 2001b).

Results

Experiment I: Effects of predators on parasite avoidance behavior and initial infection

Initial mass and Gosner stage for each species are shown in Table 6.1. Infection levels differed among species (Figure 6.2a, likelihood ratio test [LRT], $X^2 = 35.20$, $p < 0.001$) but did not change in the presence of predators (LRT, $X^2 = 0.76$, $p = 0.4$), and the species x predator interaction was not significant (LRT, $X^2 = 9.56$, $p = 0.2$). Tadpoles overall increased activity after exposure to parasites (Figure 6.2b, $F(1, 152) = 5.94$, $p = 0.02$), and a significant species x time interaction suggests that this effect depended on species identity ($F(7, 152) = 2.671$, $p = 0.01$). Leopard frogs appear to have been an exception and decreased activity after parasite exposure, although this effect was not significant in post-hoc analysis (Tukey's contrast, $z = 2.847$, $p = 0.24$). The effect of predators on activity and the predator x parasite interaction were not significant ($p > 0.2$).

Experiment II: Effects of predator cue and parasite infection on traits and survival

Initial infection levels (number of encysted metacercariae) differed among species (Table 6.1, $F(7, 69) = 15.25$, $p < 0.001$). Individual infection intensities ranged from 2 to 32 metacercariae. Five tadpoles were infected with greater than 25 metacercariae, suggesting that some error occurred during counting cercariae. However, this counting error likely did not differ among species, because the same two people counted cercariae for all species, so that no bias should have been introduced in the results.

In the analysis of survival, I included the main effects of species identity, predator, and parasite and the parasite x species and predator x parasite interactions in the final model based on AIC. Survival differed among species (Figure 6.3a; LRT, $X^2 = 47.23$, $p < 0.001$), predator presence decreased survival across species ($z = -4.189$, $p < 0.001$), and the effects of parasites on survival depended on species identity (species x parasite, LRT, $X^2 = 23.25$, $p = 0.002$). The predator x parasite interaction was marginally non-significant ($z = 1.80$, $p = 0.07$), and the coefficient was positive, suggesting that any effects of predators and parasites on survival were additive or possibly antagonistic, rather than synergistic. Reductions in survival in the parasite treatment ranged from no effect on chorus frogs and bullfrogs to 39% reduced survival in wood frogs.

Final mass decreased in response to parasite infection and predator presence and differed among species (Table 6.2, Figure 6.3b). The parasite x species and predator x species interactions were both significant, suggesting that the effects of both natural enemies on growth rates depended on species identity. Posthoc analysis revealed that predator presence significantly reduced the final mass of American toads (Tukey HSD, $p < 0.001$) and green frogs ($p < 0.001$) and had a marginally non-significant negative effect on the final mass of both *Pseudacris* spp. ($p = 0.05$) but did not significantly affect the other species ($p > 0.1$). Parasite infection decreased final mass in bullfrogs ($p < 0.001$) but did not significantly affect other species ($p > 0.1$). Predators tended to slow development rate, but this effect depended on species because of a significant species x predator interaction (Table 6.2, Figure 6.3c). The effects of parasites on development rate and all other interactions were not significant. In the analysis of behavior, activity levels decreased in response to parasite infection and predators and depended on species identity (Figure 6.3d, Table 6.2). The predator x species interaction was significant, suggesting that the effects of predators on activity also differed among species. Posthoc analysis revealed that all species decreased activity levels in response to predators significantly (Tukey HSD, $p < 0.05$) except spring peepers and bullfrogs ($p > 0.5$). The parasite x predator x species interaction was significant, but the parasite x predator and species x parasite interactions were not significant. The three-way interaction suggests that the interactive effect of predators and parasites on behavior depended on species identity. In particular, parasite-exposed toads experienced higher activity levels in the presence of predators than unexposed toads, while other species experienced lower activity levels after parasite exposure compared to unexposed tadpoles when predators were present.

Relationships among components of susceptibility across species

Post-infection survival across species was not correlated with infection levels ($p > 0.1$), which suggests that differences among species in infection establishment were not entirely responsible for differential survival. Post-infection survival was also not correlated with avoidance behavior ($t (6) = -0.76$, $p = 0.5$), which suggests that the magnitude of the behavioral response did not entirely depend on the associated risk of mortality. Log infection levels in Experiments I and II were positively correlated with each other ($t = 2.63$, $df = 6$, $p = 0.04$). Avoidance behavior was negatively correlated with Experiment II (hereafter "individual")

infection levels (Figure 6.4a, t = -3.71, df = 6, p = 0.01) but not Experiment I (hereafter "group") infection levels (t = -1.29, df = 6, p = 0.25).

Relationship between susceptibility and traits across species

I tested the hypothesis that species traits (habitat use and development rate) influence susceptibility to parasite infection using linear models. Before correction for phylogeny, the only significant effect was a positive relationship between avoidance response and variation in snail association (Figure 6.4b, F (1, 6) = 10.57, slope = 1.69, p = 0.02, R^2 = 0.64). However, the results differed when PICs were used in analysis. The final model for the parasite avoidance response included development rate and variation in associated snail density (R^2 = 0.823, F (2, 4) = 14.94, p = 0.01). Species that experience more variation in associated snail densities had the largest avoidance response (slope = 2.03, t (4) = 5.45, p = 0.006). A negative effect of development rate on avoidance behavior was marginally non-significant (slope = -0.25, t (4) = -2.33, p = 0.08). The final model for group (Experiment I) infection level included variation in associated snail densities and development rates, but this model was marginally non-significantly better than the null (F (1, 5) = 6.457, p = 0.08), although a positive effect of development rate on group infection levels was significant within the model (slope = 0.18, t = 2.91, p = 0.04). The model for individual (Experiment II) infection levels included all terms but was not significantly better than the null (F (3, 3) = 3.96, p = 0.14). In the model for survival post-infection (R^2 = 0.88, F (1, 5) = 47.26, p <0.001), only development rate was included, and the effect of development rate was significant (slope = 0.16, t (5) = 6.875, p <0.01). However, the effect of development rate on survival was not significant when wood frogs were excluded from the analysis (F (1, 4) = 0.26, p = 0.6), suggesting that wood frogs were an influential data point. No component of susceptibility had a significant phylogenetic signal (p > 0.2).

Finally, behavioral responses to predators were negatively correlated with behavioral responses to parasites (Figure 6.4c; t (6) = 3.10, p = 0.02). Species that strongly increased activity in response to parasites also strongly decreased activity in response to predators. This correlation remained significant after calculating PICs (t (5) = 3.63, p = 0.02). No other component of susceptibility was significantly correlated with the response to predators.

Discussion

The results reveal differences among species in avoidance behavior, infection levels, and the effects of infection on survival and traits, consistent with my first hypothesis that susceptibility differs among species due to trait differences. These susceptibility differences may have important implications for community structure and host-parasite dynamics. Effects on host-parasite dynamics may result from differential infection rates among species that drive dilution or amplification effects. For example, co-occurrence of wood frogs with leopard frogs may result in reduced wood frog infection rates, if highly-infected leopard frogs reduce numbers of cercariae available to infect wood frogs (i.e., a dilution effect, which has been documented in another amphibian-trematode system, Johnson et al. 2008, Johnson et al. 2013). Effects on community structure may occur due to differential post-infection survival, due to apparent competition and keystone effects. For instance, the presence of parasites may reduce the abundance of wood frog tadpoles relative to *Pseudacris* spp., due to the lower post-infection survival of wood frogs. Furthermore, trait-effects may also influence community structure, and such trait-mediated indirect effects may be of comparable magnitude to density-mediated effects (Peacor and Werner 2001). For instance, competitive interactions between bullfrogs and green frogs may change due to the differential effects of parasites and predators on growth rates.

Multiple components of susceptibility (avoidance, resistance, and tolerance) contribute to these differences among species. The lack of a correlation between infection levels and post-infection survival suggests that differences among species in tolerance or post-infection resistance (e.g., cyst elimination) contribute to differential effects of parasites on survival, rather than differences in initial infection levels alone. In addition, the negative correlation between avoidance behavior and individual infection levels (Experiment II) suggests that differences in avoidance responses contribute to differential infection levels across species, in line with a documented relationship between intraspecific infection rates and the avoidance response (Koprivnikar et al. 2012). Alternatively, species that are adapted to avoid infection behaviorally may also be adapted to resist infection physiologically (e.g., immune response and behavior could be genetically linked or involve a shared physiological pathway). Surprisingly, despite the correlation between individual and group infection rates, I did not observe a significant correlation between avoidance response and group infection levels. A possible explanation is that the relationship between a species' avoidance response and the odds of successful infection by a cercaria can depend on spatial scale (e.g., Marino and Werner, in press). In the individual

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exposures, because both the host and parasites were confined to 60 mL cups, host-parasite encounter rate was likely high regardless of activity levels, while the avoidance response probably reduced the success of infection post-contact. In contrast, in the group exposure in 8 L aquaria, increased movement as a result of avoidance behavior may have actually increased host-parasite encounter rates even while reducing the success of infection post-contact, which may have dampened the realized efficacy of the parasite avoidance response. Behavior and spatial scale may thus jointly influence infection rates.

Species differences in each component of susceptibility may have larger-scale ecological implications. Avoidance and resistance reduce infection intensities in particular hosts, which may affect the number of cercariae available to infect other amphibian hosts (e.g., through dilution effects) and have downstream effects in the transmission cycle. Tolerance may increase successful transmission to definitive hosts, because parasite-induced host death results in a dead end for the parasite. In addition, avoidance, resistance, and tolerance may impact host community structure. For instance, differential avoidance or resistance may influence the relative abundance of species through effects on infection rates, because trait and mortality effects of echinostomes are dose-dependent (Marino et al, in press).

The results indicate that host traits, including life history, habitat use, and breeding phenology, have likely influenced the evolution of parasite susceptibility. First, the significantly lower post-infection survival and the trend of higher infection levels in faster-developing species are generally consistent with a growth-mortality tradeoff (Schiesari et al. 2006) and the documented relationship between pace-of-life and susceptibility to parasitism in amphibians (Johnson et al. 2012). Second, the correlation between species' variance in association with snails and avoidance behavior suggests that habitat use may have influenced the evolution of this behavioral trait. Trematode parasite distributions are dependent on snail host distributions (Skelly et al. 2006, Johnson et al. 2013), and snail distributions depend strongly on many of the same abiotic factors that also influence the distribution of amphibians (e.g., pond area, canopy cover, fish presence, Werner et al. 2007b, Hoverman et al. 2011). Furthermore, plastic defense traits, including behavior, are hypothesized to be selectively favored in species that experience greater heterogeneity in their association with natural enemies (Gabriel et al. 2005). For instance, anuran species occurring in habitats where predator densities are more variable exhibit more plastic trait responses to predators, consistent with the adaptive plasticity hypothesis (Van

Buskirk 2002). The relationship between variability in snail associations (and thus habitat variability) and avoidance behavior is thus consistent with an adaptive plastic response to parasitism, similar to that in response to predators (Van Buskirk 2002). Interestingly, the correlation between behavioral responses to parasites and predators suggests that similar traits (e.g., life history, habitat use) may influence how species respond to both parasites and predators. In particular, spatial covariation between snail and predator abundance driven by abiotic factors (e.g., pond size, hydroperiod) may have resulted in selection for a strong behavioral response to both groups of natural enemies in the same species. Finally, breeding phenology may also have contributed to the observed differences in susceptibility among species. The three most infected species were all spring-breeding species (leopard frogs, American toads, and wood frogs), while two of the three (green frogs and bullfrogs) least infected species were summer-breeding species. In addition, post-infection mortality was greatest for a spring breeding species, wood frogs. Echinostomes and other trematodes tend to increase in abundance in mid-to-late summer (Sapp and Esch 1994, Peterson 2007, Raffel et al. 2011), so that species which breed in late summer may experience higher exposure than spring-breeders. The results are thus consistent with the hypothesis that selection may cause lower susceptibility in summer breeding species.

The results also show that species' differences in the effects of parasites can depend on community context, particularly the presence of predators. The observed three-way interaction suggests that species differ in the interactive effects of predators and parasite infection on activity levels. Reduced activity levels in the presence of parasites reduce predation risk (Anholt and Werner 1995), and reduced activity levels after parasite infection may allow tadpoles to minimize the physiological costs of parasite infection (e.g., impaired renal function, Holland et al. 2007). Consequently the interaction may represent a floor effect that differs among species, which may depend on baseline activity levels. A more interesting possibility is that the behavioral interaction could reflect differences among species in documented interactive effects of parasite infection and predator stress on physiology, e.g., the corticosterone response (Marino et al., in press), which can influence behavior, predator defenses (Fraker 2008, Middlemis Maher et al. 2013), and immune response (Apanius 1998). This explanation would be in line with my second hypothesis that predator presence differentially affects the susceptibility of species to parasites, due to differential interspecific responses to a susceptibility tradeoff. When exposed to these natural enemies in combination, species may differentially alter their behavior and

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physiological response to minimize either costs of infection or predation risk, especially if those costs can differ among species. In particular, toads act unlike other species because they are more active when exposed to the combination of natural enemies than in response to infection alone. A possible explanation is that toads are fast developing and unpalatable to predators (Relyea 2001b). Consequently, an increase in activity may have a smaller effect on predation risk (due to reduced palatability) while also allowing for increased foraging and growth to metamorphose more rapidly and escape the greater risk associated with both natural enemies.

However, contrary to my expectations, I did not find additional evidence for tradeoffs or parasite x predator interactions. The effects of predators and parasite infection on growth and survival were additive across species (consistent with previous studies, Marino et al., in review, Thiemann and Wassersug 2000, Raffel et al. 2010). Surprisingly, I found no effect of predator cues on behavior or infection levels in Experiment I, despite evidence for these effects elsewhere (e.g., Thiemann and Wassersug 2000, Relyea 2001a, Fraker 2008, Szuroczki and Richardson 2012). A possible explanation is that I used a small size class (~22 mg) that was relatively inactive (mean activity = 6.3%). I selected a small size class because they are most likely to experience negative effects of infection (Schotthoefer et al. 2003, Holland et al. 2007). However, the effects of predator cue on infection levels can depend on size class (Marino, in prep), because tadpoles' behavior changes throughout ontogeny (Brown and Taylor 1995), and effects of predator cue can depend strongly on size (Fraker 2008). Nevertheless, despite the small size class used, I did observe a general effect of cercariae addition on behavior, regardless of the presence of predator cue. My results thus suggest that small tadpoles may perceive cercariae as a greater threat than predators at this size class and time scale.

A number of factors could have influenced the findings of these experiments. A relatively low number of parasites was used relative to infecting intensities observed in some ponds (Skelly et al. 2006), which may not reveal differences in post-infection survival among species that occur at higher infection intensities, as the effects of infection are dose dependent (Marino et al., in press). Second, although there was no evidence for a phylogenetic signal in susceptibility, incorporating phylogenetic information and divergence times mattered for the analyses. Analyses using PICs calculated from the tree with branch lengths probably provide the best-supported inference here. Finally, my estimates of species' associations from southeastern Michigan may not fully represent each species' historical association with echinostomes across its range. In

addition, I only collected animals over a small geographic area, while susceptibility may differ across a species' range (as has been shown for other amphibian pathogens, Schock et al. 2009, Flechas et al. 2012).

These results suggest critical next steps to identify the mechanisms underlying observed species differences and the consequences for community structure. Comparisons among species that directly measure physiology (e.g., immunocompetence) in addition to behavior should reveal the relative contributions of these traits to observed differences in susceptibility. Furthermore, these results can be used to generate hypotheses for the consequences of species differences in parasite susceptibility and predator response for community structure (e.g., through apparent competition) or parasite transmission (e.g., through a dilution effect). Such hypotheses can be tested in future experiments that directly examine multi-host species communities.

In conclusion, these results provide important insights into the relationship between biodiversity and disease. An evaluation of the effects of other trophic levels, especially predators, on parasite-host interactions will be critical, given the role of shared traits in interactions with both natural enemies. Knowledge of the relative magnitude of parasite and predator effects and whether those effects are additive or non-additive will make the challenge of incorporating multiple natural enemies into community models more tenable. Furthermore, measuring differences in components of susceptibility and understanding how those components are interrelated will be key steps in developing a predictive model for the interdependence between community structure and disease.

Lastly, these findings have important conservation implications. Echinostome infection prevalence is associated with human activities, such as urbanization (Skelly et al. 2006) and agrochemical use (Rohr et al. 2008). Amphibians are a globally threatened class (Stuart et al. 2004), and disease is believed to play a role in those declines (Daszak et al. 2003, Vredenburg et al. 2010). Differential infection rates and effects of disease agents on hosts, and their interactions with other stressors, including predation, likely influence population-level effects of disease, which has important consequences for global amphibian diversity.

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Table 6.1: Initial Gosner (1960) stage (range) and mean mass ± SE (mg) of tadpoles used in experiments, and initial infection levels ± SE (number of metacercariae) of tadpoles used in Experiment II. I weighed 20 experimental animals to determine initial mass. I measured Gosner stage and infection levels of 10 tadpoles that had been exposed to 25 cercariae for 12-18 h simultaneously with experimental tadpoles.

Species	Gosner Stage	Mass (mg)	Infection Level (individual exposure to 25 cercariae)
Bufo americanus	26-28	20.7 ± 0.6	13.2 ± 0.7
Hyla versicolor	25-26	21.8 ± 0.8	13.1 ± 0.9
Pseudacris crucifer	25-26	23.6 ± 0.8	11.3 ± 0.1
Pseudacris triseriata	25-27	23.6 ± 0.4	8.2 ± 1.9
Rana catesbeiana	25	23.9 ± 0.9	10.5 ± 1.8
Rana clamitans	25	22.3 ± 0.9	7 ± 1.0
Rana sylvatica	25-26	25.3 ± 0.5	16.7 ± 1.3
Rana pipiens	25-26	20.6 ± 0.7	23.8 ± 2

Table 6.2: Results of ANOVAs examining the effects of echinostome infection, caged *Anax* predator presence, and species identity on log-transformed final mass (mg), log-transformed Gosner stage, and arcsine-squareroot transformed activity level (proportion of tadpoles active) of green frog (*Rana clamitans*) tadpoles over four weeks during Experiment II.

ln (Final Mass [mg])	F	df (between groups)	df (within groups)	p
Parasite	8.24	1	164	**0.005****
Predator	79.47	1	164	**<0.001*****
Species	281.00	7	164	**<0.001*****
Block	1.26	56	164	0.13
Species x Parasite	3.95	7	164	**<0.001*****
Species x Predator	5.31	7	164	**<0.001*****
Parasite x Predator	2.10	1	164	0.15
Species x Parasite x Predator	1.59	7	164	0.14
ln (Development Rate + 1)				
Parasite	3.02	1	163	0.08
Predator	113.46	1	163	**<0.001*****
Species	331.11	7	163	**<0.001*****
Block	2.37	56	163	**<0.001*****
Species x Parasite	2.3	7	163	0.1
Species x Predator	6.726	7	163	**<0.001*****
Parasite x Predator	0.089	1	163	0.9
Species x Parasite x Predator	0.45	7	163	0.7
Transformed Proportion Active				
Parasite	6.99	1	168	**0.009****
Predator	214.52	1	168	**<0.001*****
Species	60.05	7	168	**<0.001*****
Block	1.51	56	168	**0.02***
Species x Parasite	0.59	1	168	0.44
Species x Predator	6.36	7	168	**<0.001*****
Parasite x Predator	1.21	7	168	0.2
Species x Parasite x Predator	2.1	7	168	**0.046***

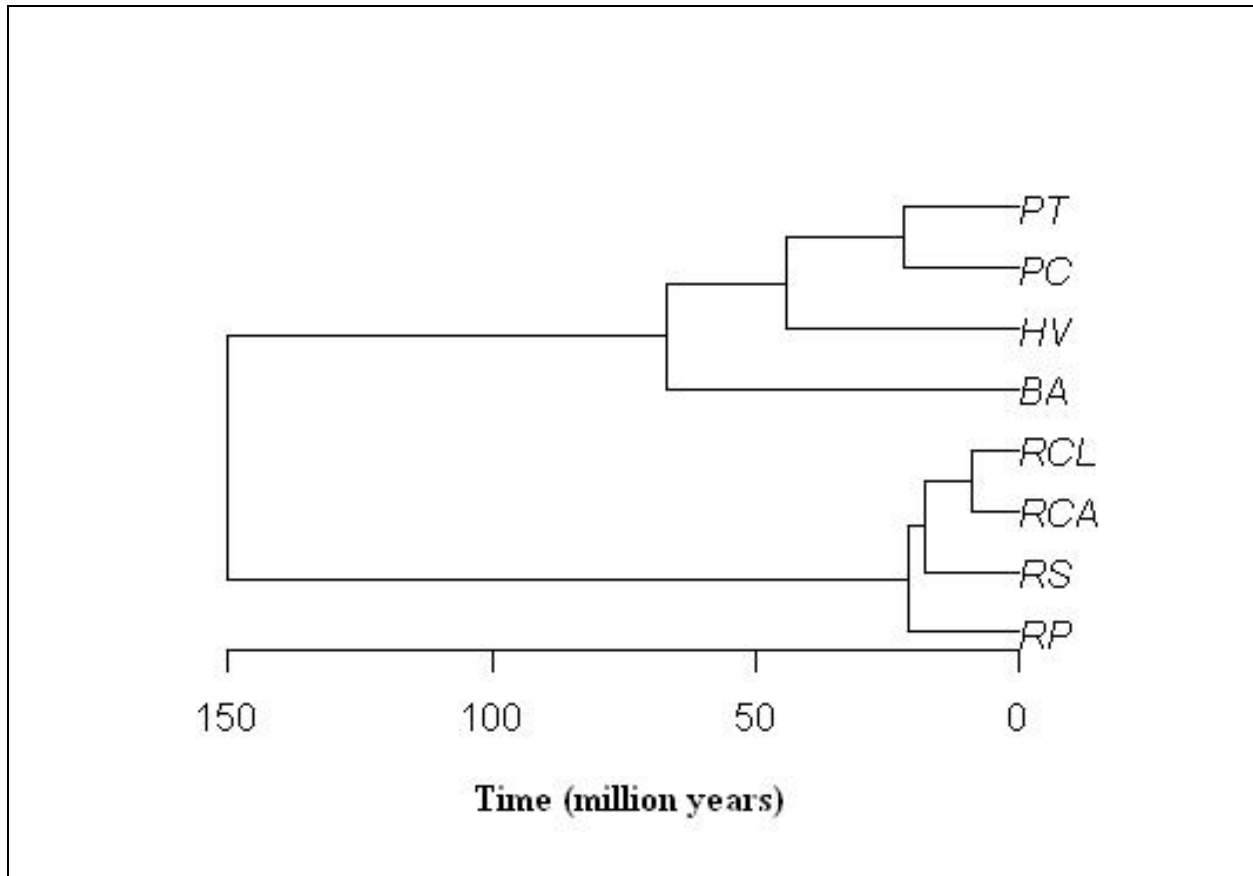Signif. codes: '***' < 0.001, '**' < 0.01, '*' < 0.05

Figure 6.1: Phylogeny of the anurans used in this study. PT = *Pseudacris triseriata*, PC = *P. crucifer*, HV = *Hyla versicolor*, BA = *Bufo americanus*, RCL = *Rana clamitans*, RCA = *Rana catesbeiana*, RS = *Rana sylvatica*, RP = *Rana pipiens*. Phylogeny was constructed based on established relationships among taxa and published divergence times (see Methods for details; Bossuyt et al. 2006, Frost et al. 2006, Hillis and Wilcox 2006).
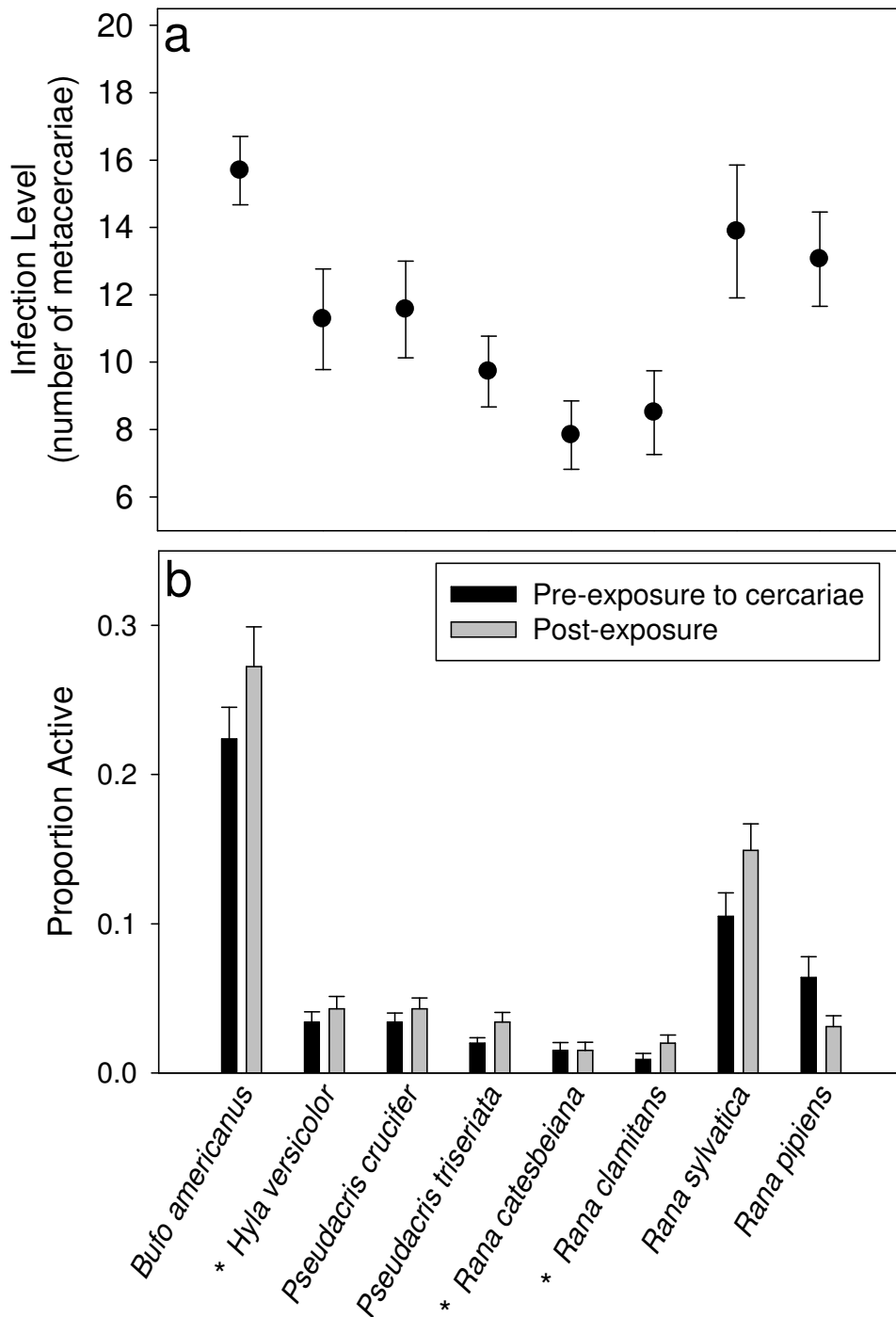
Figure 6.2: a) Mean ± SE infection levels (number of metacercariae per individual) of tadpoles of eight species of larval frogs exposed in groups of five in aquaria to 200 echinostome cercariae after 48 h in Experiment I. b) Mean ± SE activity levels of eight species of larval frogs 30 min before (black bars) and 15 min after (gray bars) addition of 200 echinostome cercariae in Experiment I. Summer breeding species are starred.
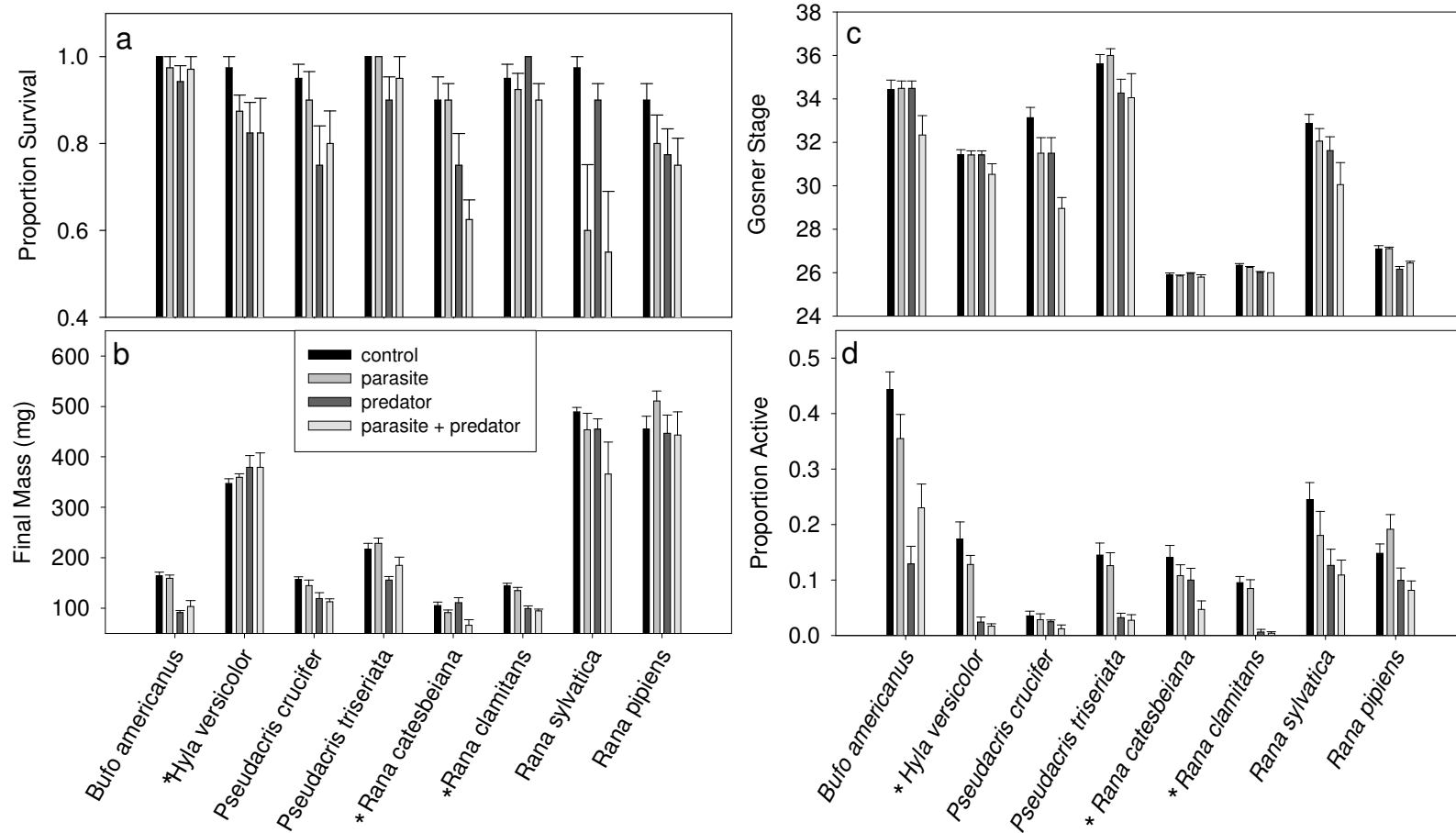
Figure 6.3: a) Mean ± SE survival (proportion alive after four weeks), b) final mass, c) Gosner (1960) stage, and d) activity level (proportion active) of eight species of larval frogs exposed or not exposed to echinostomes and then exposed or unexposed to caged *Anax* predators in Experiment II. Summer breeding species are starred.
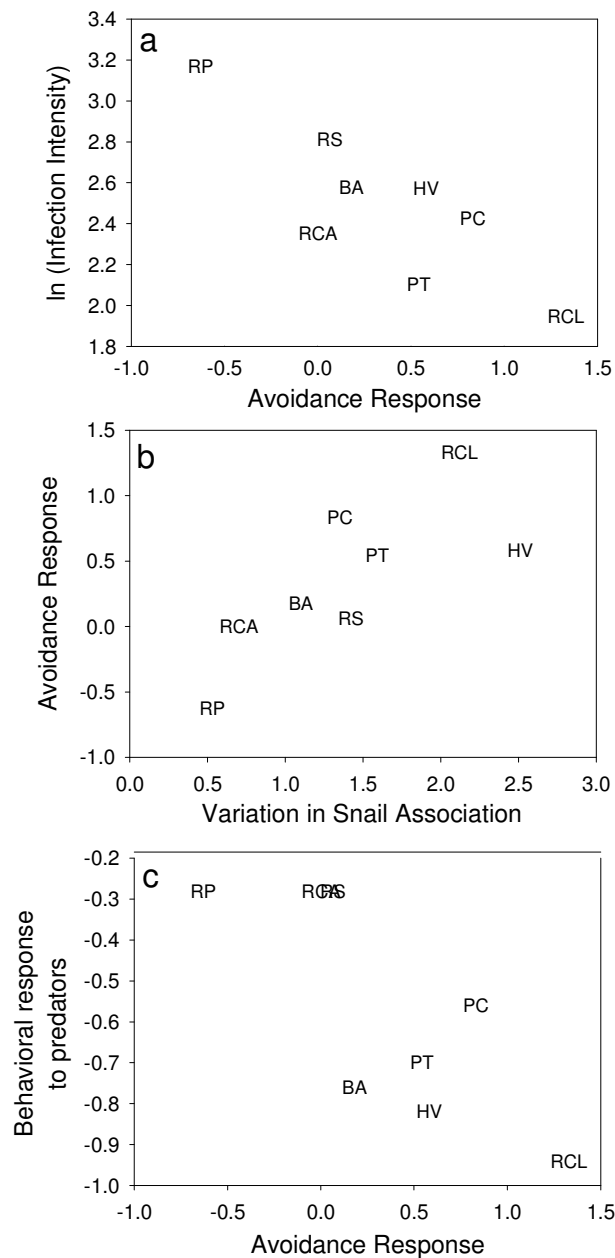
Figure 6.4: a) Log mean infection level (number of metacercariae) of individual tadpoles exposed to 25 cercariae was lowest for species exhibiting the largest avoidance response ($F_{(1, 6)}$ = 13.26, p = 0.01, R^2 = 0.69). b) Parasite avoidance response (proportional change in activity after cercariae addition) was greatest for species that have the greatest variation in their association with snail hosts ($F_{(1, 6)}$ = 8.244, p = 0.03, R^2 = 0.58). Variation was quantified as the standard deviation of snail densities in ponds in which each species occurs, based on a survey of ponds in southeastern Michigan. c) Behavioral response to predators (the proportional change in activity levels) was stronger for species that exhibited a stronger parasite avoidance response (t (6) = 3.10, p = 0.02). Species labels defined in the Figure 6.1 caption.

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Chapter VII

The distribution of echinostome parasites and consequences for larval frog survival

Introduction

Like predation and competition, parasitism can strongly influence population dynamics through effects on individual fitness (Anderson and May 1978, Hudson et al. 1998). However, parasites have historically received relatively less attention, and field studies linking parasite distributions and dynamics to host demographic rates (e.g., Hudson et al. 1998, Duffy and Hall 2008) are lacking for most animal systems. One reason for the relative paucity of studies is that parasites at endemic levels have often been assumed to play a relatively unimportant role in dynamics compared to other factors, such as predators, competition, or weather (Davidson and Andrewartha 1948, Solomon 1949, Hairston et al. 1960, Sih et al. 1985, Chase et al. 2002). In addition, key population parameters for both hosts (e.g., density of mobile animals) and parasites (e.g., infection intensity and aggregation among hosts) can be especially challenging to collect. However, despite logistical challenges, the importance of parasites at the population scale should be tested. Population-level effects of parasites may be important in many systems, given the ubiquity of parasites in food webs (Lafferty et al. 2006).

Assessing the population-level effects of parasites should benefit from a holistic approach examining the pathway from environmental context to host population dynamics. Environmental factors can control the distributions of parasites, which determine potential effects of parasites on host fitness, which in turn determine the dynamical consequences of parasitism. First, linking environmental context and parasite distributions requires understanding the contribution of factors at both the individual host and population scale, although both scales are seldom considered simultaneously. At the host scale, a number of traits (e.g., body size, behavior, immunocompetence) may influence observed distributions of parasites (Saino et al. 1995,

Wilson et al. 1996, Zelmer and Arai 1998). At the population and landscape scales, both abiotic and biotic factors can affect hosts and parasite free-living stages (e.g., Pietrock and Marcogliese 2003, Orlofske et al. 2012), with consequences to parasite distributions (Johnson et al. 2013, Richgels et al. 2013). Second, linking parasite distributions to host fitness requires understanding the relationship of host fitness and infection levels (Anderson and May 1978, Benesh 2011). Although effects of parasites may be addressed in part in laboratory studies, laboratory conditions may poorly mimic the field. Better measurements of infection and fitness in the field are badly needed. Finally, linking fitness effects to host population dynamics can depend on a range of factors, such as aggregation of parasites (Anderson and May 1978, Shaw 1998) and community context (Packer et al. 2003, Duffy and Hall 2008), that can have unexpected consequences. For example, mortality due to parasitism may be compensatory under strong intraspecific competition (Washburn et al. 1991).

Here, we take steps to develop a holistic understanding of a larval frog – trematode parasite system, linking environmental context, parasite distributions at multiple life stages and scales, and host demographic rates. Larval frogs provide an ideal system to address factors influencing the distribution of parasites and population-level effects. Tadpoles are frequently parasitized (Hoverman et al. 2012, Johnson and Hoverman 2012) and occur in discrete populations (i.e., ponds) that vary considerably in habitat characteristics (e.g., pond size, hydroperiod, canopy cover) that influence both parasite (Richgels et al. 2013) and amphibian distributions (Werner et al. 2007). We focus on echinostomes (Digenea: Echinostomatidae), which are common, generalist parasites with a complex life cycle (Kanev et al. 2000) that can cause larval frog mortality (Chapter II, Holland 2010). Evidence suggests that echinostome abundance increases near human activities, such as agriculture and urbanization (Beasley et al. 2005, Skelly et al. 2006, King et al. 2007, Rohr et al. 2008b). However, factors underlying echinostome distributions and dynamics in a natural context and the effects of parasitism at the host population scale are poorly understood. Our objectives here were 1) to characterize the drivers of trematode parasite distributions and 2) evaluate the effects of echinostomes on larval frog survival, which may contribute to population dynamics (Karraker et al. 2008). To this end, we used a combination of field surveys and a field experiment, focusing on infection and survival of larval green frogs (*Rana clamitans*) and wood frogs (*Rana sylvatica*).

Methods

Study System

Echinostomes have a complex life cycle involving three hosts (Kanev et al. 2000). Adult worms typically live in the intestinal tract of mammal or bird hosts, where they reproduce sexually. Eggs pass in the feces, hatch, and a free-swimming miracidium enters a snail host, where the parasite goes through multiple rounds of asexual reproduction during sporocyst and redia stages. Cercariae, another free-swimming form, exits the snail host and can infect a second intermediation host - a snail, fish, or larval amphibian. In larval frogs, cercariae enter through the cloaca and encyst in the kidney as metacercariae. When the second intermediate host is consumed by the definitive host, the parasite completes its development to adulthood. Echinostome infection has been shown to affect larval frog traits and reduce larval frog survival in the laboratory (Chapter II, Holland et al. 2007), mesocosms (Chapter III, IV), and field experiments (Holland 2010).

Field survey of infection in snails and larval green frogs

To evaluate factors that influence echinostome distributions among ponds, we surveyed infection in 23 ponds on the Edwin S. George Reserve (ESGR) in Livingston County, Michigan (Figure 7.1). Ponds were selected from a set of 37 ponds based on survey data which indicated where snail hosts had recently occurred (Hoverman et al. 2011). These 37 ponds were part of a long-term survey in which amphibian, predator, and snail densities as well as a range of abiotic factors were measured (Werner et al. 2007, Werner et al. 2009, Hoverman et al. 2011).

Ponds were sampled in June and July, 2008, and monthly from May-August from 2009-12 with dip nets (22×27 cm, 1×2 mm mesh size). Our surveys were performed mid-month and were simultaneous or within one week of the long-term survey, except during August because the long-term survey occurred only May-July. We assessed infection in five snail species, as some echinostome species can exploit multiple species of snail hosts (Kanev et al. 2000, Detwiler et al. 2010). We collected *Planorbella trivolvis*, *Stagnicola elodes*, and *Physa gyrina* on all dates and *Planorbella campanulata* and *Helisoma anceps* from 2010-12. In July and August 2010-12, we also collected green frog tadpoles, which were euthanized and preserved in 70% ethanol. We collected 20 individuals of each snail species and 10 tadpoles from each pond on each date when possible, but we discontinued sampling after 20 person-minutes in small ponds (<750 m^2), 30 person-minutes in medium ponds (750-1500 m^2), and 40 person-minutes in large

ponds (>1500 m^2). On several dates, few or no snails or tadpoles were collected from some ponds due to low densities or if the pond was dry. We determined a pond as having too low a density to continue sampling when we were unable to collect 5 individuals of any focal species after at least 10 person-minutes of sampling.

All snails were screened for parasite infection by placing them in 60 mL water in sample cups and putting them under a 60 W light for a minimum of 3 hours. Due to space limitations, typically multiple (2-3) snails of the same species from a single pond were initially put into a single cup together. After 3 hours, all cups were examined under a dissecting microscope for the presence of trematode cercariae. If any trematode cercariae were observed before at least 3 hours elapsed, the snails were separated into new individual cups and placed under a 60 W light for at least two additional hours. Any snails which did not release cercariae after at least 3 hours were recorded as not infected. After the additional 2 hours, cups containing individual snails were examined under a dissecting microscope to examine if any trematode cercariae were present. If present, we moved a sample of cercariae into a small Petri dish in 70% ethanol. The cercariae were then identified as echinostomes or other groups based on morphology (Schell 1985). We note that this method for assessing infection likely underestimated actual snail infection, as any infected snails which did not actively shed cercariae were counted as not infected. All snails were preserved in 70% ethanol. To assess amphibian infection, five tadpoles (or all tadpoles, when fewer) from each pond on each date were dissected under a microscope. We dissected the mesonephri, nephric ducts, and mesonephri and counted the number of echinostome metacercariae present.

Infection rates and survival in pond enclosures

We used enclosures to measure infection and survivorship of tadpoles in a subset of eight ponds (including one off-ESGR pond) that varied in infection prevalence and snail densities. Due to the long breeding season, tadpoles collected during the survey may have experienced a range of exposure times to parasites, and also may have experienced selective predation which could influence patterns in observed infection intensities. Enclosures allowed us to exclude predators and control for initial size of tadpoles and length of time in ponds. Green frog (*Rana clamitans*) egg masses were collected from the experimental ponds on the Edwin S. George Reserve (ESGR) during the first week of June, 2010, and placed in 300 L pools filled with aged well water. After hatching tadpoles were fed ad libitum with Purina ® Rabbit Chow until the

beginning of the experiment. On June 22, 2010, 10 sets of 10 tadpoles (21.4 ± 0.9 mg, Gosner (1960) stage 25) were placed in 0.5 L plastic containers of water from the culture pools and placed into the pond for 30 minutes, in order to acclimate to pond temperature. Enclosures (30 x 45 x 5 cm) were constructed of window screening covering a frame of plastic fencing. Ten green frog tadpoles were added to each cage along with three pieces of polystyrene to ensure floatation near the pond surface, and the cage was closed with two plastic zip ties. Pairs of cages were secured with zip ties to five stakes (~1.5 m PVC pipe, diameter = 2.54 cm) which were used to hold each cage into place, with a total of 10 cages per pond. The five stakes were placed at least 5 m from the edge of each pond, and at least 5 m apart from one another. In ponds with multiple habitat types (open water, submergent vegetation (e.g., *Elodea sp.*), emergent vegetation (e.g., cattails and water lilies), or overhanging vegetation), we arranged the stakes so that some cages would be in different microhabitat types. One half of the cages (one from each pair on each stake) were collected after two weeks. Remaining cages were collected after four weeks. After cages were collected, tadpoles were removed, counted, weighed, euthanized, and preserved in 70% ethanol for later dissection.

Parasitism and survival to metamorphosis

To evaluate the population-scale effects of parasites, we examined the relationship between infected snail density and wood frog survival from egg to metamorphosis. Wood frog survival was measured in six natural ponds on the ESGR as part of a long-term study of wood frog metapopulations (Benard et al., in prep). Briefly, wood frog egg masses were counted in each pond immediately following the breeding season each year, which typically occurs over a few days in late March-early April in southeastern Michigan. Subsets of egg masses were photographed and the eggs in each mass counted, in order to estimate initial egg mass densities in each pond. Each pond was enclosed by a drift fence (~36 cm height). Bucket traps (4 L buckets, with a ~9 x 9 cm square holes in the lid for animals to fall into) were buried approximately every 10 m around the perimeter inside of the fence to capture wood frog metamorphs as they emerged from the pond. Fences were closed before wood frog metamorphs began emerging in mid-June and were checked twice daily until all metamorphs had emerged from the ponds, typically by early July. Metamorphs were collected, counted, marked using an injectable elastomer dye, and a subset weighed as they emerged from the pond, and all were then released. Analyses from the comprehensive data reveal strong evidence of negative density

dependent survival between egg and larval stage and no effect of predators on survival (M. Benard, pers. comm.). Here, we examined a subset of data for which parasite data were available to test for an effect of parasites. Snail density and prevalence data was available for three of these ponds for the years 2008-2010, except only two years (2009-10) for one pond. The remaining three ponds have low snail densities and infection data was not collected, so we excluded those ponds from our analysis here.

Species-level identification

We used molecular methods to identify the echinostome species present at our field site. We dissected snails and removed five rediae or sporocysts, and we then extracted DNA using a Qiagen DNeasy Tissue Extraction Kit and amplified a portion of the 28S ribosomal DNA using digenean-specific primers (Dig12 and LSU 1500R). We confirmed successful amplification using gel electrophoresis and the PCR product (1/5 dilution) was submitted to the University of Michigan DNA Sequencing Core for sequencing. We examined chromatograms for each sequence in Sequencher and compared sequences to those of known species using NCBI Nucleotide BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Statistical Analysis

All analyses in this study were performed in the R statistical package v.2.15 (http://www.r-project.org). For the survey data, we analyzed the factors that influence snail and green frog infection using mixed models with year and pond as random factors. We generated models using combinations of potential predictors that we expected could influence infection (e.g., due to effects on definitive host visitation, host-parasite contact rates, or snail demographics), and the final model was determined using AIC. We analyzed snail infection prevalence using a generalized linear mixed effects model (GLMM) with a binomial distribution, using the glmer function in the lme4 package (http://cran.r-project.org/web/packages/lme4). Initial predictors included snail species identity, day in year, pond canopy cover, hydroperiod, and maximum pond area (log-transformed). We also included the species x day interaction in the model, because snail species differ in life history traits that may influence how infection prevalence changes seasonally. For a subset of dates (2008-2010) for which additional data were available, we performed additional analyses including total snail density (CPUE) and hydroperiod (proportion of days dry) as additional predictors. We also performed Mantel tests to examine whether infection prevalence in each snail species and overall was spatially correlated,

using the mantel.rtest function in the ade4 package (http://cran.r-project.org/web/packages/ade4). For the Mantel tests, we calculated prevalence across years using data from snails collected during June and July.

We analyzed green frog infection intensities using GLMM with a negative binomial distribution implemented using the glmmADMB package (http://glmmadmb.r-forge.r-project.org). Fixed factors included individual size (snout-vent length, SVL), developmental stage (Gosner 1960), snail infection prevalence, year, and month sampled, with pond as a random factor. For a subset of dates for which additional data were available (2009-10), we also performed additional analyses that included more predictors, including infected snail density (CPUE x snail infection prevalence), anuran densities (CPUE), and predator biomass (per m^2).

For the enclosure experiment, we analyzed larval frog infection and survival of tadpoles using mixed models. In the analysis of infection, we used a GLMM with a negative binomial distribution and included microhabitat, infected snail density, and date as potential fixed factors, with random effects of enclosure, stake, and pond. Infected snail density was calculated as the product of July snail density and annual snail infection prevalence during 2010. We analyzed survival using GLMM with a binomial distribution, with mean infection in enclosures as a fixed factor and random factors of site and pond.

In the analysis of wood frog survival, we examined whether survival, mass at metamorphosis, and larval duration depended on the density of infected snails using linear models, with pond identity as an additional predictor. Infected snail densities were calculated as the product of May snail density (concurrent with larval wood frog occurrence in ponds) and annual infection prevalence.

Results

Field survey of infection in snails and larval green frogs

Of the 8,700 snails collected, 458 were infected with echinostomes, so that overall echinostome infection prevalence was 5.3%. Echinostomes were found in snails in all but one pond, and infected tadpoles were recovered from that pond and all other ponds from which tadpoles were collected. Echinostomes are thus ubiquitous among ponds containing appropriate snail hosts on the ESGR, although prevalence within and among ponds varies extensively over time. In the snail infection analysis, day in season, snail species, and hydroperiod were included

in the final models based on AIC. Infection prevalence overall tended to increase throughout the summer (z = 5.429, p<0.001), and species differed in infection prevalence (Table 6.1; X^2 = 42.33, df = 3, p <0.001). A significant species x day interaction suggests that the seasonal change in infection prevalence differed among species (Figure 7.2; X^2 = 35.00, df = 3, p <0.001). Infection prevalence also tended to be greater in ponds that were drier for more of the year (z = 2.59, p = 0.01). We found no evidence for a correlation between infection and spatial distance between ponds for any species or across species (Mantel tests, p > 0.1).

Of 250 green frog larvae collected from 16 ponds on the ESGR, 48 were not infected. Our analysis included data from 206 tadpoles, which were collected from ponds and on sampling dates for which we collected >15 snails (i.e., to ensure a sufficient sample size to provide a reasonable estimate of snail infection prevalence). The distribution of infection levels was strongly skewed to the right (median = 11 metacercariae, maximum= 555 metacercariae). Snail infection prevalence, SVL, and collection year were included in the final model based on AIC. Infections in larval green frogs increased with snail infection prevalence (Figure 7.3a; deviance = 8.46, df = 1, p = 0.003) and SVL (Figure 7.3b; deviance = 62.23, df = 1, p <0.001).

Infection rates and survival in pond enclosures

Survival was lower in cages that had higher cage mean metacercariae per host (Fig 7.4a, z = -2.3, p = 0.02) and did not differ across dates (X^2 = 0.0054, p = 0.94). Infection intensities of tadpoles in enclosures differed among ponds (deviance = 14.51, p < 0.001) and between dates (z = 4.3, p < 0.001), increasing from mean ± SE = 3.2 ±1.2 metacercariae per tadpole on the first date to 8.0 ± 2.3 on the second date. Infection was lower in enclosures in open portions of the pond compared to other microhabitats (Fig 7.4b, z = -1.96, p = 0.05). There was not a significant relationship between infection and infected snail density across sites (deviance = 0.018, p = 0.9).

Parasitism and survival to metamorphosis

Wood frog survival decreased with increased densities of infected snails (Figure 7.5; p = 0.02). Infection prevalence for one pond in one year was based on only 4 sampled snails. However, the negative relationship between infected snail density and survival remains significant even after removing that point (p=0.04), which increases the minimum sample size to 30 snails. Survival also differed among ponds (F (2, 4) = 12.45, p = 0.02). There was no relationship between infected snail density and mass at metamorphosis or larval duration (p > 0.1).

Species-level identification

Echinostome DNA was amplified and sequenced from 21 infected snails (4 *P. trivolvis*, 8 *S. elodes*, 7 *P. gyrina*, and 2 *P. campanulata*) collected across the ESGR. Sequences from echinostomes collected on the ESGR closely matched (>99% sequence similarity for all but 1 sample that was 98% similar) three genera: *Echinostoma*, *Echinoparyphium*, and *Euparyphium* (Table 7.S1). Matches for *Echinostoma* and *Echinoparyphium* occurred in all four snail species. Matches for *Euparyphium* occurred only in *S. elodes*, and different genera occurred in different snail species within the same pond.

Discussion

Our results provide insights into the links between environmental context, parasite distributions, host fitness, and host demographics, furthering a holistic understanding of the echinostome-snail-amphibian system. We identified factors that influence parasitism over time (seasonality) and at the host (i.e., snail species, tadpole size), microhabitat, and pond (i.e., hydroperiod) scales across multiple hosts in the parasite life cycle (i.e., snails and amphibians). Further, we provide evidence that, consistent with laboratory and mesocosm experiments, parasite infection in the field can impact host survivorship, detectable at the population scale. These relationships may have important implications for effects of parasites on host population dynamics, community structure, and parasite-mediated natural selection.

Our results reveal potential hotspots for parasite transmission to amphibians at multiple scales. Among ponds, high snail infection prevalence unsurprisingly increases larval green frog infection. Within ponds, infection was greatest in tadpoles in enclosures in structured microhabitats, likely due to higher snail densities in those areas. Structured microhabitats are typically preferred by larval frogs because of protection from predators and higher resource levels (Warkentin 1992, Tarr and Babbitt 2002), but parasitism may introduce a potential cost. The result of spatial heterogeneity at these scales may be altered parasite dynamics (Paull et al. 2012) and natural selection on hosts' response to parasites, such as spatial avoidance of parasites by tadpoles (Rohr et al. 2009) or adult breeding site selection (Kiesecker and Skelly 2000).

Other observed patterns may result from multiple mechanisms and have additional important dynamical and evolutionary implications. First, seasonal variation in snail infection with echinostomes, which has also been documented elsewhere (Sapp and Esch 1994, Peterson

2007), may result from seasonal variation in snail mortality, reproduction, and definitive host visitation. Seasonality in snail infection contributes to within-population variation in larval frog infection (Raffel et al. 2011) and will be important to consider in predicting parasite dynamics (Altizer et al. 2006). For instance, a consequence of seasonality in snail infection is that breeding phenology mediates amphibian parasite exposure levels - spring-breeding frogs (e.g., wood frogs) experience lower echinostome exposure than summer-breeders (e.g., bullfrogs, *Rana catesbeiana*). Second, increasing snail infection with hydroperiod may result from increased contact rates between miracidia and snail hosts in ponds with dropping water levels or due to reduced snail population turnover in those ponds (e.g., due to fewer invertebrate predators present in ponds and years with shorter hydroperiods, Werner et al. 2009). An implication is that frog species which occur in ponds with longer hydroperiods (e.g., northern leopard frogs, *Rana pipiens*) likely experience lower parasite exposure, due to reduced infection prevalence in snails (although snail density must also be taken into account). Third, variation among snail species in infection prevalence and dynamics may result from differential resistance to infection (Raberg et al. 2009) or differences in contact rates with miracidia originating from habitat use (Hoverman et al. 2011). The result is that the degree of habitat overlap between amphibians and certain snail species influences amphibian host-parasite dynamics, so that infection dynamics may be more tightly synchronized for some snail-amphibian associations (e.g., green frogs and P. trivolvis typically overlap, Werner et al. 2007, Hoverman et al. 2011) than others. Finally, higher infection levels in larger tadpoles may result from longer durations of exposure for older, larger tadpoles and also higher infection rates in large tadpoles and lower survivorship of smaller tadpoles with high infection intensities (Holland et al. 2007, Chapter IV). Aggregation in larger hosts may allow for enhanced persistence of the parasite, given that larger hosts are better able to tolerate infection (Holland et al. 2007) and probably experience lower mortality generally.

Our parasite survey findings thus complement existing knowledge of environmental factors influencing snail and amphibian distributions (e.g., pond size, fish presence; Werner et al. 2007, Hoverman et al. 2011) to identify the conditions that enhance associations between amphibians and parasites. Knowing the strength of echinostome-amphibian associations will be important for predicting the effects of species composition on parasite dynamics (e.g., Johnson et al. 2013) and evolution of defenses against parasites, such as avoidance behavior (Chapter VI) and the physiological response of hosts to parasites (Middlemis Maher et al. 2013, Chapter II).

Despite the relationships noted above, much variation in infection remains unexplained. Several factors that we hypothesized could influence definitive host visitation (e.g., pond size) and host-parasite contact rates (e.g., host density) provided no explanatory power. Unmeasured variation in individual host traits (e.g., immunocompetence) and stochasticity in definitive host visitation and host-parasite contact rates at multiple life stages likely contribute to unexplained variation, which future research should elucidate. In addition, differences among echinostome species may contribute to some of the observed spatial and temporal variation and merit further research.

Our results also suggest that parasitism reduces host survivorship, with potential consequences for host populations. Survivorship was negatively associated with echinostome infection or abundance in both individual green frogs in enclosures and wood frogs at the population level. Echinostomes have been demonstrated to reduce larval frog survivorship in experimentally-exposed animals in previous experiments (Holland et al. 2007, Holland 2010, Chapters II-VI), but our results are the first to directly relate natural exposure to reduced larval survivorship (but see Beasley et al. 2005 for evidence of a similar pattern observed in cricket frogs, *Acris crepitans*, in largely human-impacted ponds). A negative effect on wood frog survival might be counterintuitive, given that wood frogs breed earlier in the season and metamorphose relatively rapidly and thus tend to miss peak exposure periods. However, wood frogs were the most susceptible of Michigan frog species in the laboratory (Chapter VI), so that even moderate exposure levels may reduce survivorship. This result suggests that there are indeed negative effects of parasites on survival, as expected, and these effects are probably additive or depensatory, rather than compensatory. Notably, an effect of parasites on wood frog survival was detected, while no evidence has been found for a negative effect of predators (M. Benard, pers. comm.). While predators are typically thought to play an important role in larval frog survivorship, parasites may thus play a comparable or even greater role in some circumstances.

However, alternative explanations for differential survival should be noted, as conditions under which parasites thrive may be poorer conditions for tadpoles generally. For instance, other parasites and pathogens occur in some of the surveyed ponds (e.g., *Ribeiroia ondatrae*, *Batrachochytrium dendrobatidis*), which occur at lower prevalence (Zellmer et al. 2008, Marino et al., unpublished data), but could have affected survival if study tadpoles were exposed and

infected. In particular, trematode parasite distributions can be correlated in amphibian hosts (Johnson and Hoverman 2012), likely because snail distributions are correlated and the same snail host species is used by multiple parasites. For instance, we have observed 8 morphologically distinct trematode cercariae from *P. trivolvis* on the ESGR, several of which are known to infect amphibians.

Nevertheless, our results are at least suggestive that echinostomes play an important role at larger scales, which may have both ecological and evolutionary implications. Larval mortality may be especially important for amphibian population dynamics (Vonesh and De la Cruz 2002, Karraker et al. 2008) and potentially influences the relative abundance of species through apparent competition, as species differ in susceptibility (Chapter VI, Rohr et al. 2010). Effects on larval survivorship may also have important feedbacks to parasite population dynamics, if parasites have a regulatory role in amphibian populations or if amphibian hosts are a limiting step in the parasite life cycle. Effects of echinostomes may interact with other stressors, such as predation (Chapter III) or competition (Chapter IV), to amplify mortality, which should be addressed in future field studies. Evidence of fitness costs of parasitism in the field also indicates the potential for parasite-mediated natural selection, which may play a role in species-level differences in susceptibility (Chapter VI) and suggests the potential for local adaptation.

Finally, these findings also may have important conservation implications, as echinostomes are strongly associated with human activities (Skelly et al. 2006, King et al. 2007, Rohr et al. 2008a), and disease is believed to play a role in recent global amphibian declines (Stuart et al. 2004). Given that we found evidence for effects of infection on survival in a natural setting, where infection rates are likely lower than developed areas, parasite effects may be greater in populations where infection prevalence is elevated due to anthropogenic stressors (Skelly et al. 2006, Rohr et al. 2008b). Future work examining links between environmental factors, parasite distributions among hosts, and the consequences for host fitness and vital rates in more disturbed habitats should be informative. Furthermore, linking these patterns with amphibian and parasite populations across both host and parasite life cycles are needed to evaluate longer term dynamical consequences.

Acknowledgements

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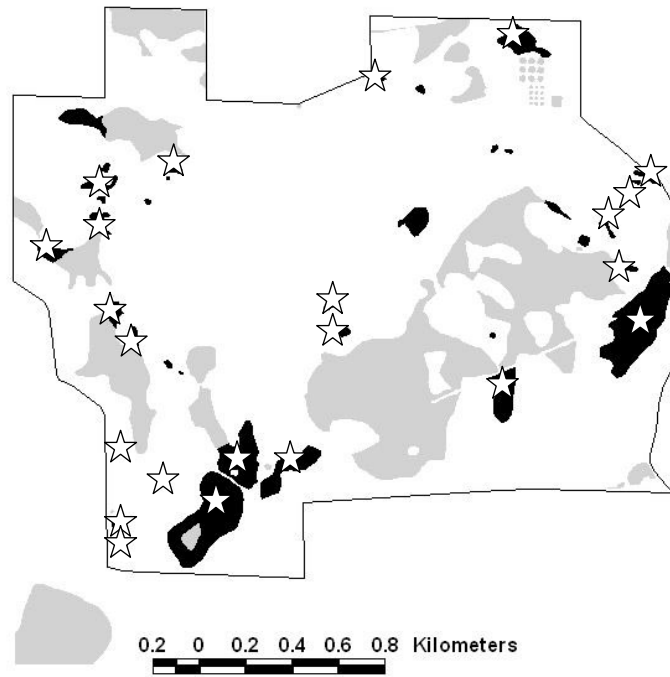
Figure 7.1: Map of surveyed ponds on the Edwin S. George Reserve in Livingston County, MI. Ponds that were surveyed for echinostomes in snails and larval green frogs (when present) are starred.
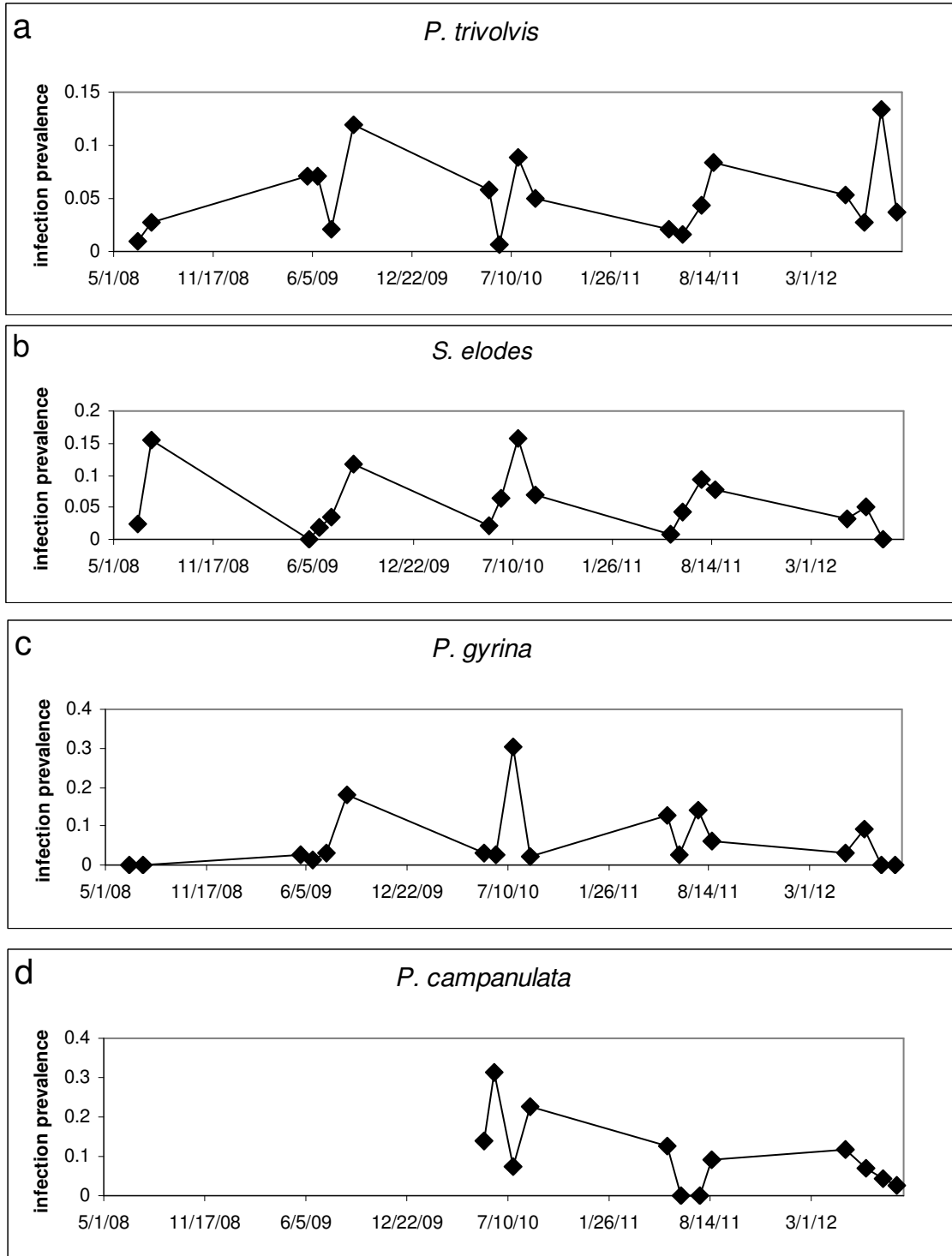
Figure 7.2: Echinostome infection prevalence (%) among ponds in four snail species on the Edwin S. George Reserve over the five-year sampling period. Twenty-three ponds were sampled monthly May-August. *P. campanulata* was sampled beginning May 2010.
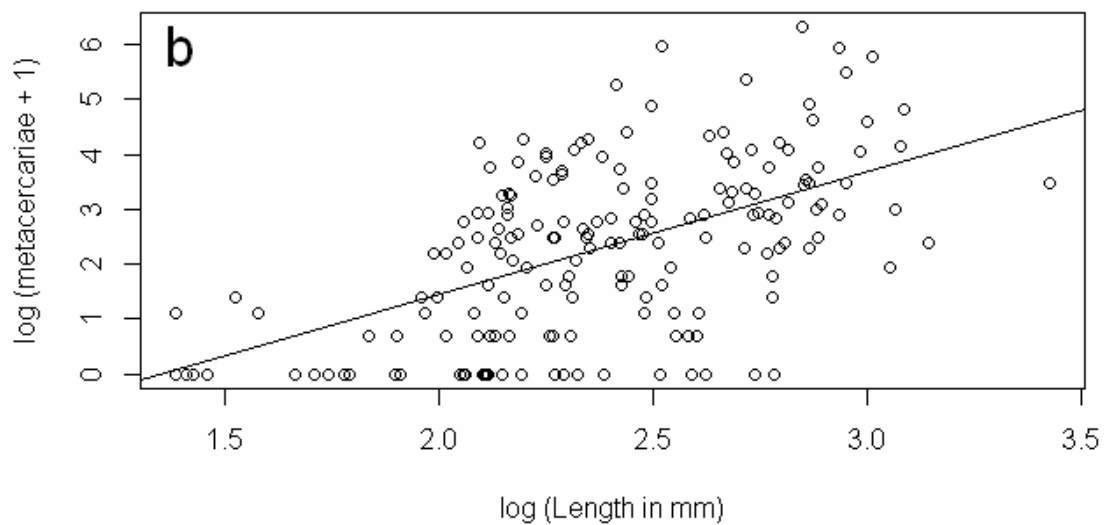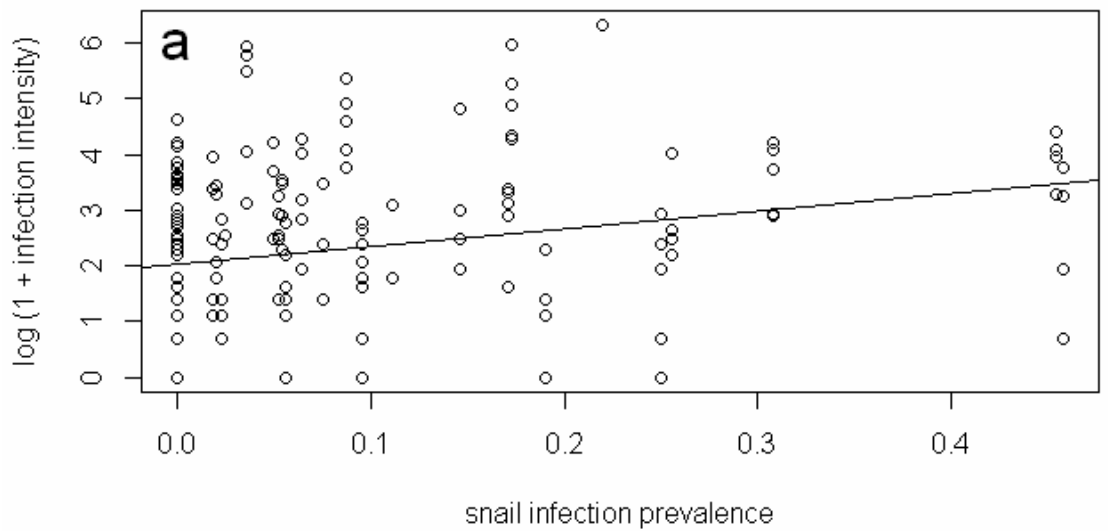
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Figure 7.3: a) Infection in individual field-collected green frog tadpoles increased with snail infection prevalence (slope = 3.18, R^2 = 0.06, t (204) = 3.58, p < 0.001). b) Infection (log [1 + number of metacercariae]) of tadpoles also increased with snout-vent length (slope = 2.23, R^2 = 0.30, t = 9.255, p < 0.001).
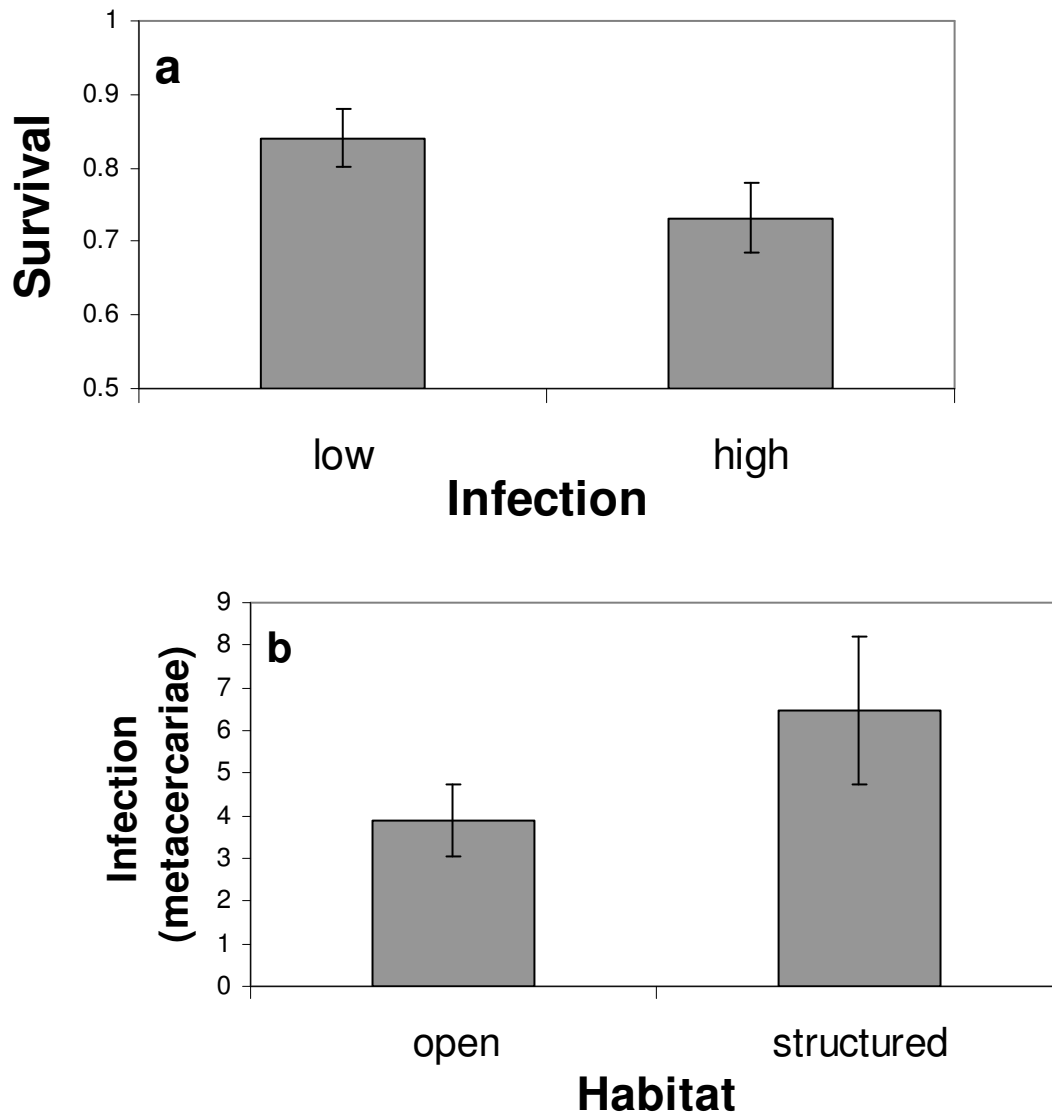
Figure 7.4: a) Larval green frog survival was lower in pond enclosures where surviving tadpoles had higher infection burdens (low: mean < 1 metacercaria, N = 26; high: mean infection ≥ 1 metacercaria, N = 31). b) Infection (number of metacercariae) was lower in tadpoles in enclosures in open areas of the pond than enclosures in structured habitat (i.e., submerged, emergent, or overhanging vegetation; p = 0.05).

Figure 7.5: Wood frog survival to metamorphosis (log-transformed) was lower in ponds and years in which density (log +1 transformed) of infected snails was higher (slope = -22.15, R^2 = 0.5997, t = -2.998, p = 0.02).

Appendix

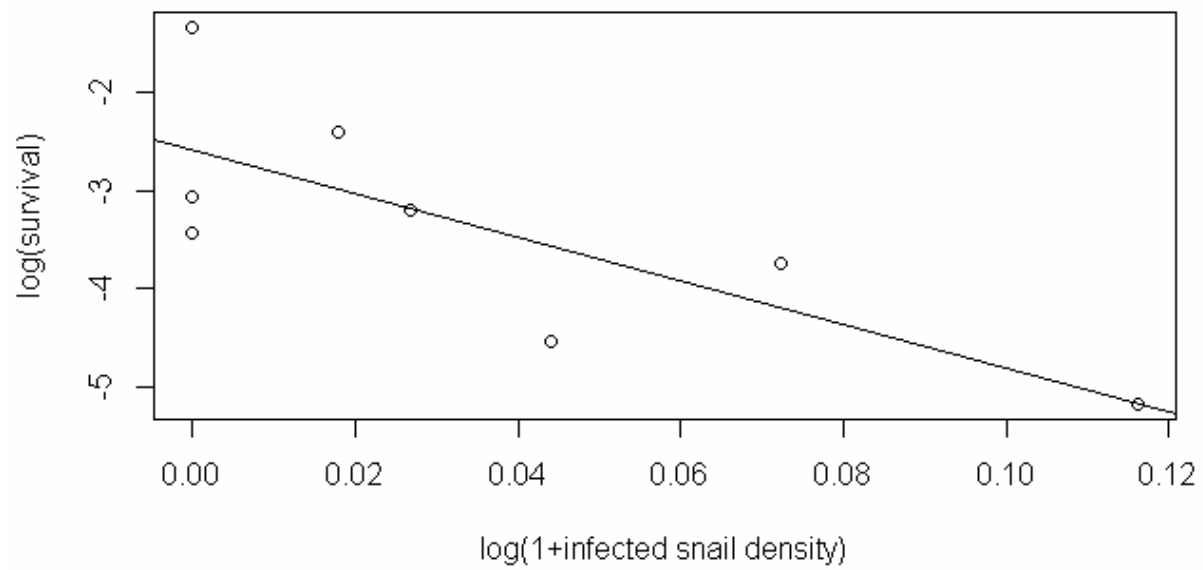**Table 7.S1**: Echinostome species identifications in 21 snails collected from ponds on the ESGR, using molecular methods.

Parasite Species	Pond	Date	Snail Species	Percentage Match
Echinostoma paraensei	Crane Pond	9-May-12	*Planorbella trivolvis*	99%
Echinostoma revolutum	Crane Pond	9-May-12	*Planorbella campanulata*	99%
Echinostoma revolutum	Crescent Pond	22-Aug-11	*Stagnicola elodes*	99%
Echinoparyphium rubrum	East Marsh	18-Jun-12	*Physa gyrina*	99%
Echinoparyphium rubrum	East Marsh	18-Jun-12	*Planorbella campanulata*	99%
Echinostoma revolutum	East Marsh	22-Aug-11	*Planorbella trivolvis*	99%
Echinoparyphium cinctum	Fishhook Marsh	18-Jun-12	*Physa gyrina*	99%
Echinoparyphium cinctum	Fishhook Marsh	22-Aug-11	*Stagnicola elodes*	99%
Echinoparyphium cinctum	George Pond	18-Jun-12	*Physa gyrina*	99%
Euparyphium melis	Gravel Pit Marsh	17-Jun-11	*Stagnicola elodes*	99%
Echinostoma revolutum	Southeast Marsh	22-Aug-11	*Planorbella trivolvis*	99%
Echinoparyphium cinctum	Spring Pond North	23-Jul-12	*Physa gyrina*	99%
Echinoparyphium cinctum	Spring Pond North	10-May-12	*Stagnicola elodes*	99%
Euparyphium melis	Spring Pond South	18-Jun-12	*Stagnicola elodes*	99%
Echinoparyphium cinctum	Southwest Swamp	17-Jun-11	*Physa gyrina*	99%
Echinoparyphium cinctum	Southwest Swamp	18-Jun-12	*Stagnicola elodes*	99%
Echinoparyphium rubrum	West Marsh #11	22-Aug-11	*Planorbella trivolvis*	100%
Echinoparyphium cinctum	West Marsh #6	10-May-12	*Physa gyrina*	99%
Echinoparyphium cinctum	West Marsh #6	15-Jun-10	*Stagnicola elodes*	99%
Echinoparyphium cinctum	West Marsh #11	17-Jun-11	*Stagnicola elodes*	98%
Echinoparyphium cinctum	West Marsh Dam Pond	18-Jun-12	*Physa gyrina*	99%

Chapter VIII

Conclusion

As shown in the preceding chapters, parasites have a broad range of effects on hosts, with important implications across scales. Echinostomes reduced larval frog survivorship in laboratory, mesocosm, and field experiments, and field survey results suggest that parasite effects contribute to a key population-level parameter, survival to metamorphosis. Parasites also affected important functional traits, including growth, development, behavior, morphology, and physiology. Parasite effects were often comparable in magnitude to those of predators or competitors, highlighting the critical role that parasites can play in dynamics and food webs, despite a relative lack of attention.

My findings from both experimental and survey work also demonstrate how host-parasite interactions depend on environmental context, which is vital to assess the overall effects of parasites on hosts. Fieldwork results indicated how environmental context (seasonality, pond hydroperiod, snail species distributions) influences the association between parasites and amphibian hosts. Experimental work in both the laboratory and mesocosms suggest how ecological context influences the effects of parasites on hosts. In particular, two major influences on larval anuran communities, predation and competition, affect and are affected by interactions with parasites, with implications for multi-host species communities and population dynamics in natural ponds.

Parasites and predators

Most animals experience threats from parasites and predators, and my results demonstrate several mechanisms through which the combination jointly affects shared victims. In Chapter II, I showed how parasites and the nonconsumptive effects of predators jointly affect individual traits. The trait responses of larval frogs to parasites depended on the presence of predators, including physiological, behavioral, morphological, and developmental responses. These traits mediate interactions between larval anurans and both natural enemies, as well as competitive

interactions, and the observed effects may have important carryover effects into later amphibian life stages (Relyea 2001). In addition, the observed interactions suggest that larval frogs experience tradeoffs in responding to multiple natural enemies (e.g., a physiological tradeoff mediated by CORT).

In Chapter III, I demonstrated larger-scale consequences of the combination of natural enemies for larval frog survivorship, including both consumptive and nonconsumptive predator effects. After finding a synergistic effect of predators and parasites on survival in mesocosms, I then identified the contribution of likely potential mechanisms to the observed synergism. These mechanisms included trait effects of parasites on predation risk, trait effects of predators on parasitism, and density-mediated effects of predators on parasitism. The results suggested that tadpoles' parasite-avoidance response contributed to increased predation risk and the observed synergism, due to increased visibility of more active tadpoles to predators. Thus, larval frogs experience a potential tradeoff between responding to predators and parasites, with consequences for important vital rates (i.e., survivorship). Surprisingly, other effects observed at a small scale, such as nonconsumptive effects of predators on infection intensity (Thiemann and Wassersug 2000) and interactive effects of the combination of natural enemies on traits (e.g., developmental rate) were not apparent at the larger scale.

As in the tadpole-echinostome system, tradeoffs between responding to different groups of natural enemies are likely common and broadly important (Sih et al. 1998, Hatcher et al. 2006), given the shared role of many traits in host-parasite and predator-prey interactions. Behavioral (Kats and Dill 1998, Moore 2002), physiological (Dhabhar 2009, Middlemis Maher et al. 2013), and life history traits (Minchella 1985, Benard 2004) are involved in the response of many animals to both groups of natural enemies. Identifying potential tradeoffs and the involved traits will be a useful framework to predict potential interactions between natural enemies in other systems. Furthermore, using similar approaches to those here may provide broad insights into the interactive effects of natural enemies. Examining consequences of predation risk across the entire host-parasite interaction timeline and systematically evaluating the mechanistic basis for interactions, rather than focusing on a single time point or mechanism, should provide advances in understanding such interactions generally.

Parasitism, competition, and host resources

Host-parasite interactions often occur in the presence of host competition or resource limitation, which can cause interactive effects (Coop and Kyriazakis 1999). In the tadpole-echinostome system, my findings in Chapter IV suggest that competition can modify the effects of parasitism on host fitness, including growth and survival. In addition, the findings in Chapter V showed that resource levels may mediate the relationship between infection (scaled to host mass) and survival, and resource levels can strongly depend on competitive context. These results thus suggest a potential tradeoff between responding to competition and parasitism. Competition may alter resource intake, assimilation, or allocation that influences host defenses against parasites.

The results in these chapters also demonstrate complex influences of competition and resource levels on parasite transmission. Chapter IV revealed that increased host density can result in increased infection at the mesocosm scale, which is consistent with potential stress effects of competition (Glennemeier and Denver 2002) that can increase infection with parasites (Belden and Kiesecker 2005). In contrast, Chapter V showed that increased resources can increase infection, due to a positive effect on growth and size-dependent infection rates. Together, my results thus emphasize the importance of considering the balance between counteracting effects of competition on traits (e.g., growth and physiology) that influence infection.

Parasitism in multi-host contexts

Differential effects of parasites on host species can affect community structure (Hudson and Greenman 1998) and parasite transmission (Johnson and Thieltges 2010). In addition, comparisons of susceptibility among species can provide insights into the evolutionary response of hosts to parasites (Johnson et al. 2012). In Chapter V, I showed differences among anuran species in infection rates and the survival and trait effects of parasites. Species' differences in susceptibility were associated with habitat use, developmental rate, and breeding phenology, which provided novel evolutionary insights. For instance, I found that the adaptive behavioral response of tadpoles to parasites correlates with variability in habitat use, consistent with the adaptive plasticity hypothesis. Adaptive plasticity may thus be a generally useful concept in addressing variation among species in host-parasite interactions, as for predator-prey interactions (Van Buskirk 2002). The observed differences among species also likely have important

consequences for community structure, mediated both through direct effects of parasites as well as the interactions with predators and competition discussed in earlier chapters.

Parasitism in natural ponds and scaling up

The potential implications of individual-level effects of parasites at the population scale have been extensively modeled and discussed (Anderson and May 1978, May and Anderson 1979, Dobson and Hudson 1992), yet the hypothesized effects are relatively rarely tested empirically. In Chapter VII, I show that larval frog mortality rates in the field are associated with parasitism, consistent with much of the preceding laboratory and mesocosm experimental work. Undoubtedly, a next step will be to evaluate the consequences of other effects examined experimentally, such as synergistic effects of parasites and predators, in natural populations, which will require more data. Nevertheless, the result that endemic levels of parasitism influences survivorship provides an important first step toward identifying population effects.

Comparing the results at the aquarium, mesocosm, and population scales reveals the challenges of scaling up individual-level effects, and the importance of considering how potentially conflicting roles of individual traits play out across scales. For example, parasites can have both positive and negative effects on individual hosts' activity levels, depending on whether the tadpole is engaged in an avoidance response to cercariae or is experiencing the pathogenic effects of infection, respectively. The expectations with respect to predation risk, which increases with activity level (Anholt and Werner 1995), are thus difficult to establish. The results of our first mesocosm experiment in Chapter III indicate that the avoidance response ultimately predominated, resulting in an overall amplification of predation risk at the mesocosm scale. Similarly, the opposing effects of host size on infection rates and tolerance of infection make prediction of overall fitness consequences difficult without an explicit test of effects at a larger scale. Thus, a comprehensive understanding of these interactions could only be gained by examining the role of these traits at multiple scales.

Tackling context dependence

The strong context-dependence of host-parasite interactions is not surprising, but it raises troubling issues about the ability to generalize in ecology. Repeatedly finding evidence for context dependence across studies examining a range of parameters (e.g., predation risk, density, host size, spatial scale, resource levels, etc.) suggests that understanding host-parasite systems is

a many-dimensional problem. Additional complications (e.g., nonlinear functional relationships) may make the problem seemingly intractable.

Attempts to simplify the problem may allow for progress. For example, narrowing the research focus to only hypothesized important traits and interactions may make gaining insights more tenable. A useful approach, demonstrated here, is to evaluate the key functional traits involved in host-parasite interactions, and then identify environmental factors that likely have strong effects on those traits or a strong effect on host-density. Identifying likely trait-mediated tradeoffs, e.g., between susceptibility to parasites and predators, should be helpful to identify those key traits. In the case of larval frogs, much previous research demonstrated the importance of predators and competition for larval frog traits and survival (e.g., Wilbur and Collins 1973, Peacor and Werner 2001, Relyea 2004). Furthermore, some key traits involved in echinostome-tadpole interactions (i.e., size and activity level) were known (Koprivnikar et al. 2006, Holland et al. 2007), which my research expanded upon. I was able to exploit this background to develop and test specific hypotheses for potential interactions, based on knowledge of individual-level effects. The results were predictable in some cases (e.g., synergistic effects of predators and parasites) but revealed additional complexities elsewhere (e.g., parasitism x competition interactions), which motivated further research resulting in further insights (e.g., the complex relationship between growth rate and infection). Thus, although my results confirm that context dependence is likely to be a common and important issue, the approach used here provides a framework to move forward.

A note on conservation implications

Although my overarching goal was to examine the effects of echinostomes in a natural context, I hope that the insights gained will also inform an understanding of the effects of echinostomes on amphibian populations affected by anthropogenic activities. Other researchers have shown that human activities can increase the abundance of echinostomes (e.g., Skelly et al. 2006, Rohr et al. 2008), which likely impacts amphibian communities. My research adds to this understanding by demonstrating how additional stressors modify parasite effects, which will be important to assess the overall consequences of higher parasite abundances. These findings also provide general insights into combined effects of disease and other stressors, which is of general relevance to amphibian declines globally (Collins and Storfer 2003). My findings thus may have

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important policy and land management implications (e.g., regulation of non-point source pollution, wetland conservation).

Future Directions

My results suggest a number of valuable avenues for future research. Below, I describe three areas that I think will be particularly fruitful to address going forward.

Cross-life stage and cross-generational consequences of parasitism

The effects of parasites on amphibians may have consequences for frog demographics across life stages and generations, as larval stressors can have downstream effects on hosts after metamorphosis (Relyea 2001, Groner et al. 2013), and larval mortality is a key demographic rate in amphibian population dynamics (Vonesh and De la Cruz 2002, Karraker et al. 2008). Future work should examine the effects of parasites, coupled with the additional stressors examined, across life stages (e.g., effects of larval parasitism and predation on juvenile mass and condition). In addition, our findings likely have longer term dynamical consequences that should be addressed in further research. For instance, developmental and physiological effects of parasites and interactive effects of multiple stressors on larval frog survivorship likely affect amphibian dynamics across years and generations. Stage-structured population models parameterized using field and experimental measurements of parasite infection and fitness effects will be useful to inform host-parasite dynamics over longer timescales, and the consequences of parasite effects relative to and in combination with other factors, such as predation and competition.

Effects of traits and community context on parasite transmission and persistence (e.g., resource levels) also likely have longer-term consequences for parasite distributions and dynamics. However, links to natural dynamics and distributions are difficult, because the role of the definitive host in driving patterns and dynamics in natural populations is unknown. Future work to gain insight into important parameters such as definitive host visitation and transmission rates could provide key links allowing a more complete understanding of parasite dynamics across its life cycle. Admittedly, such a task is daunting given the unknown and potentially broad definitive host use of echinostomes. However, recent methodological advances offer a possible alternative. Population genetics tools may allow inferences into parasite dispersal and transmission, even in the absence of data for all life stages (Prugnolle et al. 2005). Such approaches may help close the parasite life cycle loop, allowing for a better understanding of the

overall consequences of effects of community context on single parasite life stages and the landscape-scale processes influencing their distributions.

Population-level variation in susceptibility and local adaptation

In addition to ecological implications, context-dependence and fitness effects of natural enemies may have important implications for eco-evolutionary dynamics (Carius et al. 2001, Cousyn et al. 2001, King et al. 2011, Duffy et al. 2012). My comparative results demonstrate extensive variation among species in susceptibility associated with traits. However, the degree of intraspecific genetic variability in susceptibility, and its dependence on community context, is unknown. Building on my results here, I hypothesize that the combination of parasites and predators may influence population-level variation among ponds, due to local adaptation, which can have strong effects on population dynamics (Walsh et al. 2012).

For this future work, wood frogs are an ideal candidate system to address population-level differences, because they typically breed in their natal ponds (Berven and Grudzien 1990), although the likelihood of dispersal to other breeding ponds increases from lower quality natal habitats (e.g., high predator densities, M. Benard, pers. comm.). Their high philopatry partly explains why wood frogs exhibit population genetic structure at relatively small geographic scales (<5 km, Newman and Squire 2001, Zellmer and Knowles 2009) and allows populations to adapt to local conditions (Skelly 2004), likely including predators (Relyea 2002). Predator-mediated local adaptation, which has been documented in ranids (Van Buskirk and Arioli 2005), is probable in wood frogs, because even adjacent ponds can differ greatly in predator densities, selection by predators favors defensive traits (Van Buskirk and Relyea 1998), and these traits are heritable (Relyea 2005). Local adaptation in response to echinostomes is also likely. Wood frog populations from neighboring ponds can experience large differences in their association with echinostomes, because echinostome distributions in larval amphibians are limited by snail distributions and abiotic factors (Chapter VII). Wood frogs are the most susceptible of the eight common local Michigan species to echinostomes (Chapter VI), and selection is likely in natural ponds, given evidence for survival effects of parasites (Chapter VII). Due to interactions documented here (Chapters II and III), it is unlikely that the adaptive responses of populations to parasites and predators occur independently. Instead, natural selection by these natural enemies likely occurs interactively, with potentially important ecological consequences (e.g., altered parasite transmission, predation rates, and community structure).

Parasitism in multi-host communities

Differences between species in parasite infection and tolerance likely matter in multi-host communities, due to potential effects of species composition on parasite transmission (LoGiudice et al. 2003, Johnson et al. 2013) and of parasites the relative abundance of species (MacNeil et al. 2003, Johnson et al. 2008). In addition, differences among species in interactive effects of parasites and other stressors (i.e., synergistic effects of predators and parasites on survival, interactive effects of competition and parasitism) also likely have community-level consequences. The measured differences among species here can be used to generate predictions of parasite effects on community structure, and the consequences of community structure for parasite transmission. These predictions and the contribution of key traits (e.g., behavior, growth rates, immunocompetence) and environmental factors (e.g., competition, species richness) to parasite host interactions can then be tested experimentally. Finally, experimental results can then be compared with patterns in variation in infection, demographic rates, and community structure in natural ponds.

Conclusions

Overall, I hope that this work engenders a deeper recognition among ecologists of the key role that parasites can play across contexts and scales. My results suggest potentially important effects of parasites on individuals, populations, and communities, with additional evolutionary and conservation implications. My findings also reveal how community context, especially the presence of predators and competitors, can mediate these effects. The findings in this dissertation thus comprise important advances in integrating parasites into the broader framework of community ecology.

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