ENVIRONMENTAL (NATURAL AND ANTHROPOGENIC) EFFECTS ON HOST–PARASITE (SNAIL–TREMATODE) INTERACTIONS

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Abstract: This dissertation explored environmental effects on host–parasite interactions. Chapter 2 was published in *Ecotoxicology* and assessed the effects of the herbicide atrazine on freshwater snails. The results indicated that snails are affected by atrazine at the subcellular and cellular levels. However, effects at those levels were not transitive to effects on individual snail fitness or on snail populations. Chapter 3 was published in *Journal of Parasitology* and explored the dose–response effects of trematodes on snails. The results indicated that as the trematode egg dose increased, the probability of snail reproduction and survival decreased; however, the probability of establishing an infection increased. Chapter 4 was published in *Parasitology Research* and addressed the effects of atrazine on trematode transmission and host–parasite interactions. The results showed that atrazine reduced infected snail survival at higher atrazine concentrations, resulting in fewer cercariae being produced. On top of that, atrazine reduced the infectivity of worms to the final host. Combined, these effects reduced the transmission of an amphibian trematode. Chapter 5 was published in *Evolutionary Ecology Research* and was a common garden experiment assessing the effects of environment on snail shell morphology. The shell shape of offspring from stream and wetland snails converged within a single generation whereas shell size took 2 generations to converge, suggesting the two ecomorphs are a single species. Sequence data confirmed they were the same species. Chapter 6 was published in *Journal of Morphology* and assessed the interactive effects of the environment and parasitism on snail shell morphology. The results showed that the environment was the major driver of snail shell morphology. Parasitism played a secondary role and only affected shell crush resistance in stream snails. Trematode communities were significantly different between wetland and stream snails but the effect on shell crush resistance was not driven by differences in trematode communities.

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CHAPTER I

INTRODUCTION

Parasitism is a symbiotic cost–benefit relationship between two species where one species (i.e., the parasite) must live within-or-on the other species (i.e., the host) in order complete its life cycle (Roberts et al. 2009). Parasites, including organism that are parasitic for at least one stage in their life cycle, are estimated to make up half of the species on Earth (Price 1980) and can exhibit greater biomass than vertebrate predators in some ecosystems (Kuris et al. 2008). As a result, parasitism is thought to affect the life history of nearly every known organism (Price 1980). By definition, parasites exhibit a cost to their hosts. These costs typically include induced changes in host biochemistry and physiology, damage to cells and tissues, behavioral modifications, and/or morphological abnormalities (Thomas et al. 2005). The sheer number of parasites species, the contribution of parasites to the free-standing biomass, and their effects on hosts, makes parasites major contributors to the functions and dynamics of ecosystems (Hatcher et al. 2012; Kuris et al. 2008; Preston et al. 2013).

Although parasites exhibit negative effects on their hosts, certain parasites have been considered positive indicators of ecosystem health because they depend on host populations, food webs, and interacting host communities to complete their life cycles (Hudson et al. 2006; Marcogliese et al. 2005). This is particularly the case for digenean trematodes (Blanar

et al. 2009; Lafferty 1997) which exhibit complex life cycles and can be affected directly during free-living stages (Pietrock and Marcogliese 2003) or indirectly via effects on hosts (Patz et al. 2000). In general, trematodes exhibit a 3-host life cycle typically including a vertebrate definitive host (i.e., the host in which the parasite matures), an obligate molluscan first-intermediate host (i.e., required host where development occurs), and an obligate invertebrate or vertebrate second-intermediate host (Shoop 1998) (Fig. 1.1).

Within the definitive host, eggs are passed with host feces; in the aquatic environment, eggs hatch and become free-swimming miracidia which actively penetrate the molluscan first-intermediate host; alternatively, egg-bound miracidia are ingested by the first-intermediate molluscan host; within the mollusc — typically a snail — a miracidium will develop into a sporocysts and/or rediae; after several weeks of asexual reproduction, the snail will begin to shed hundreds or thousands of cercariae; cercariae are either free-swimming and actively encyst in/on the next host or are non-motile and must be ingested by the second-intermediate host to encyst; upon encystment, the worm develops into a metacercaria which must be ingested by the definitive host to complete the life cycle (Shoop 1998) (Fig. 1.1).

Given the complexities of trematode life cycles, very few trematode life cycles have been completed in the laboratory (Loker 2010). Thus, it is not surprising that environmental effects on host–parasite interactions have been thoroughly explored in trematode systems. Additionally, and despite the contribution of trematodes to ecosystems and concerns over the loss of trematode biodiversity (e.g., Dunn et al. 2009; Russell et al. 2015), parasite ecology and the biology of host–parasite interactions has largely been ignored and understudied (Gómez and Nichols 2013; Holmes 1993; Rózsa 1992; Thompson et al. 2010).

The focus of this dissertation is to understand how environmental factors, including natural and anthropogenic, affect host–parasite interactions. The aims of my first three chapters were to understand how atrazine, a commonly used herbicide, affected hosts, parasites and their transmission, and host–parasite interactions (including interactive effects of atrazine and parasitism on hosts, the production of parasites from hosts, and parasite infectivity to hosts). In CHAPTER II, I focused specifically on atrazine toxicity to snails because snails are obligate hosts for the nearly all of the 24,000 trematode species (Dobson et al. 2008; Shoop 1998). I conducted acute and chronic toxicity tests of atrazine on four species of freshwater snails (*Biomphalaria glabrata*, *Helisoma trivolvis*, *Physa acuta*, and *Stagnicola elodes*) and incorporated a literature review on the effects of atrazine on freshwater snails. I chose to use four species because atrazine effects could be species specific. Alternatively, atrazine could affect various snail species in a similar way. Thus, when evaluating the effects of atrazine on host–parasite interactions, my choice of snail species could affect the outcome of my snail–trematode system. Although I use four different snail species in this chapter, most studies focus on a single snail species, a single endpoint (often on a different level of biological organization; e.g., subcellular vs. survival), and use different atrazine concentrations. As a result, the literature is disjointed and it is unclear whether atrazine has the potential to affect snails at environmentally-relevant concentrations. The goal of my literature review was to bring together my research and all previously published work on the effects of atrazine on snails in a way that addresses species-specific effects and effects at different levels of biological organization (i.e., from genetic- to community-level effects).

The next step in evaluating the effects of atrazine on host–parasite interactions was to address the reality that snails experience variation to trematode exposures in nature. Snails could experience high trematode exposures that could result in host death, whereas low parasite exposure could result in an unsuccessful infection (Sorensen and Minchella 2001). The goal of CHAPTER III was designed to better understand how to quantitatively expose snails to different trematode egg doses resulting in snail cercariae production without "over-infecting" and killing the snail host. This was a critical step to address chemical effects on host–parasite interactions because it helped identify variation that could be affected by atrazine. For example, atrazine would not affect host–parasite interactions if the snail was over-infected and died. Instead, atrazine could affect host or parasite life history characteristics at intermediate levels of parasite exposure where there was variation in the probability of host survival and the probability of infecting a host. For this experiment, I chose to use the trematode, *Halipegus eccentricus*. Culturing techniques to complete the life cycle of *H. eccentricus* in the laboratory have been developed, providing an exceptional model for studying the impacts of extrinsic factors on trematode transmission (Bolek et al. 2010). *Halipegus eccentricus* exhibits a 3- to 4-host life cycle including true frog (i.e., Ranidae) definitive hosts, obligate snail (*Physa* spp.) first-intermediate hosts, and a variety of obligate microcrustacean species as second-intermediate hosts (Bolek et al. 2010). Additionally, several odonate species can serve as paratenic hosts (Bolek et al. 2010; Wetzel and Esch 1996).

Next, I conducted experiments with the aim to understand atrazine effects of host–parasite interactions. Thus far, laboratory experiments attempting to isolate pesticide effects on trematode transmission have focused on the hatching success of miracidia (e.g., Raffel et

al. 2009), the production of cercariae (e.g., Hock and Poulin 2012), cercarial longevity (e.g., Koprivnikar et al. 2006), and host immunosuppression (e.g., Christin et al. 2003). Additionally, studies on cercariae released from snails typically use naturally infected snails collected in the field with an unknown pesticide exposure history. Although these studies, along with others, have all identified specific effects on hosts or specific effects on parasites, simultaneous exposures to hosts and parasites — the environmentally-relevant condition — are lacking. To overcome this deficiency, in CHAPTER IV, I assessed the impacts of the herbicide atrazine on the transmission of the amphibian trematode, *H. eccentricus*, from egg to metacercaria, simultaneously exposing intermediate hosts and trematodes to environmentally-relevant atrazine concentrations throughout the life cycle.

The second part of my dissertation focuses on natural environmental effects on hosts, parasites, and host–parasite interactions. The aims of the last two chapters of my dissertation were to understand the environmental effects on host morphology, parasite distributions, and parasite-induced morphologies. For over a century, it has been known that habitat affects snail shell morphology (Crandall 1901). Over the last few decades, it has become clear that specific effects such as water current (Trussel et al. 1993) and parasitism (Krist 2000) can induce specific shell characteristics. However, it is not clear whether environmental or biological factors are the primary drivers of snail shell morphology of whether multiple factors interact to affect snail morphotypes.

Snails in the family Physidae are known for their plastic shell morphologies (Brönmark et al. 2011) and are notoriously difficult to identify (Wethington and Lydeard 2007). Wethington and Lydeard (2007) determined that Physidae species richness was over-estimated by 50% based on shell morphologies alone. Not only is it critical to explore the

effects of various factors on snail shell morphology to determine correct species but snails also depend on their shells for protection and survival (Vermeij 1995). While sampling local streams and wetlands for snails, I observed that physid snails in wetlands were significantly larger than physid snails in streams. Additionally, after factoring out shell size, wetland snails had different shell shapes than stream snails. This led me to hypothesize if there were two distinct *Physa* species segregated based on their ecological niches or if the snails were from a single species and the environment was creating distinct ecomorphs. To date, there have been several field studies identifying different shell characteristics between habitats but none have explored phenotypic convergence or divergence in the laboratory. In CHAPTER V, I conducted a common garden experiment where I raised two generations of offspring from wetland and stream snails in a common environment. I then assessed the shell size and shell shape of wild, F1, and F2 snails using geometric morphometrics. By factoring out the effects of their original environments, and by using several methods to determine the snail species, I was able to explore the ways habitat can affect snail morphotypes, which is critical for understanding host–parasite interactions in field studies.

In CHAPTER VI, I examined the interaction of environment and parasitism on snail shell morphology. One of the most common — and most commonly overlooked — factors affecting snail phenotypes is parasitism by digenetic trematodes (Krist 2000; Sorensen and Minchella 2001). Although several studies have identified parasite-induce changes in shell size (i.e., reduced growth or gigantism; e.g., Rothschild 1936; Wilson and Denison 1980) and/or impacts on shell shape and ornamentation (Hay et al. 2005; Krist 2000; Żbikowska and Żbikowski 2005), few studies have addressed actual changes in shell shape and function in different environments (Thieltges et al. 2009). If trematode parasitism has a unidirectional

effect on shell size, strength, or shape, then the host could be maladapted to certain environments resulting in reduced transmission. In contrast, trematodes could cause snails to express unique phenotypes based on their environments, which could increase their probability of transmission. In CHAPTER VI, I conducted a field study and complementary laboratory experiment to address the effects of trematode parasitism on functional shell characteristics (size, crush resistance, and shape) of *Physa acuta* snails in flow and non-flow environments. For the field study, I used naturally infected and non-infected snails collected from wetlands (i.e., nonflow) and streams (i.e., flow). Additionally, I conducted a complementary laboratory experiment to better isolate the effects of parasitism and water flow using experimentally-infected and non-infected snails and flow and nonflow tanks.

Ultimately, the goal of this dissertation was to explore how the environment affects host–parasite interactions under environmentally-relevant scenarios. The first section of my dissertation focuses on chemical effects whereas the second section focuses on habitat effects. Understanding chemical effects on host–parasite interactions in nature can be quite difficult given the complexities of parasite life cycles and the spatial and temporal variation of chemical exposures to hosts and parasites. The experiments I conducted in CHAPTERS II–IV build up to ultimately elucidate the effects of an anthropogenic chemical (i.e., atrazine) on snail–trematode interactions, specifically focusing on snail fitness and parasite transmission under environmentally-relevant concentrations. Understanding habitat effects on host–parasite interactions can also be difficult given the many correlated factors within habitats that can affect hosts, parasites, or both. Thus, to understand how parasitism and habitat characteristics affect host–parasite interactions, I used a combination of field studies

and complementary laboratory experiments. CHAPTERS VI builds upon CHAPTER V to elucidate the roles of habitat and parasitism on host snail shell morphology.

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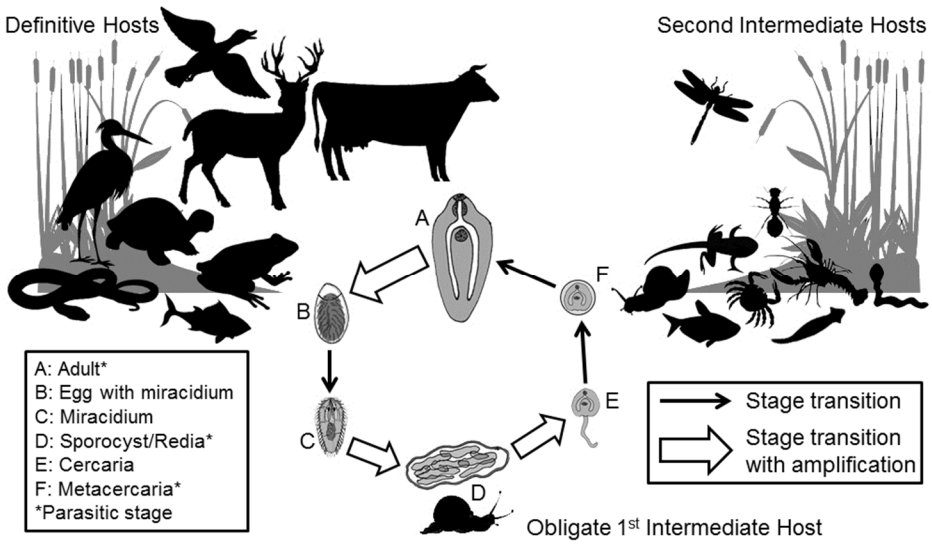

Figure 1.1. Typical three-host life cycle of freshwater trematodes (see legend for parasite stages). Trematodes commonly use fish, amphibians, reptiles, birds, or mammals as definitive hosts and a diverse array of vertebrate and invertebrate organisms as second intermediate hosts. Despite this complexity, all freshwater trematodes must return to molluscs to complete their life cycle.

CHAPTER II

THE EFFECTS OF THE HERBICIDE ATRAZINE ON FRESHWATER SNAILS

Gustafson KD, Belden JB, Bolek MG (2015) The effects of the herbicide atrazine on freshwater snails. Ecotoxicology 24:1183-1197. doi:10.1007/s10646-015-1469-x

This chapter has been published in the journal *Ecotoxicology*. The research was original and primarily conducted under the advisement of my committee member Jason Belden. My advisor, Matthew Bolek, also contributed to the research.

ABSTRACT

Atrazine has been shown to affect freshwater snails from the subcellular to community level. However, most studies have used different snail species, methods, endpoints, and atrazine exposure concentrations, resulting in some conflicting results and limiting our understanding. The goal of this study was to address these concerns by (1) investigating the acute and chronic effects of atrazine on four species of freshwater snails (*Biomphalaria glabrata, Helisoma trivolvis, Physa acuta, and Stagnicola elodes*) using the same methods, endpoints, and concentrations, and (2) summarizing the current literature pertaining to the effects of atrazine on freshwater snails. We conducted a 48 h acute toxicity test with an atrazine concentration higher than what typically occurs in aquatic environments (1,000 µg/L). Additionally, we exposed snails to environmentally relevant atrazine concentrations (0, 0.3, 3, and 30 µg/L) for 28 d and assessed snail survival, growth, and reproduction. We also summarized all known literature pertaining to atrazine effects on freshwater snails. The literature summary suggests snails are often affected by environmentally relevant atrazine concentrations at the subcellular and cellular levels. These effects are typically not transitive to effects on survival, growth, or reproduction at the same concentrations. Our acute exposures corroborate the general trend of no direct effect on snail populations as atrazine did not directly affect the survival of any of the four snail species. Similarly, environmentally relevant concentrations did not significantly affect the survival, growth, or reproduction of any snail species. These results indicate that, in the absence of other possible stressors, the direct effects of environmentally relevant atrazine concentrations may not be realized at the snail population level.

INTRODUCTION

Snails are ubiquitous members of aquatic communities and have multiple roles in aquatic ecosystems. They are primary consumers and decomposers, and serve as a major food source for a variety of vertebrate and invertebrate predators (Dillon 2000). Snails are also obligate first intermediate hosts for trematodes which cause diseases in humans, livestock, and wildlife (Pointier 1999; Shoop 1988). Given the importance of snails in nutrient cycling, food webs, parasite transmission, and their developing role in the field of ecotoxicology (Rittschof and McClellan-Green 2005), it is important to understand the impacts pesticides have on snails.

Atrazine is used to control unwanted grasses and broadleaf plants primarily in corn, sorghum, and sugarcane fields, and has been in production since the 1950s (Solomon et al. 1996). In the United States, the highest concentrations are found in the Corn Belt, generally including Kansas, Nebraska, Iowa, South Dakota, Minnesota, Wisconsin, Michigan, Illinois, Indiana and Missouri. Atrazine is applied to agricultural fields typically preemergence and ends up in aquatic systems primarily through runoff (Graymore et al. 2001; Solomon et al. 1996) Atrazine is detected in wetlands, lakes, rivers, and streams with concentrations ranging from 0.2 – 1,000 μg/L, with most concentrations occurring well below 100 μg/L (Graymore et al. 2001; Van Der Kraak et al. 2014). A recent review by Van Der Kraak et al. (2014) suggests atrazine concentrations typically do not occur at concentration >100 μg/L for longer than 14 days (<1% probability) and rarely occurs at concentrations of >20 μg/L (<5% probability). Thus, exposures to high concentrations for long periods of time are unlikely. Nonetheless, atrazine's long half-life (43-237 d) and moderately high solubility (33,000

µg/L) make it a concern for impacts on aquatic ecosystems (Graymore et al. 2001; Huber 1993; Rohr and McCoy 2010; Solomon et al. 2008).

Recent research has focused on the potential impact of atrazine on snail populations and communities (Baxter et al. 2011; Baxter et al. 2012; Rohr et al. 2012) because of the role gastropods play in aquatic parasite (i.e., trematode) transmission (Koprivnikar and Walker 2011; Rohr et al. 2008a; Rohr et al. 2008b). For example, Rohr et al. (2008b) hypothesized atrazine can change the balance of phytoplankton and periphyton resulting in an increase in the abundance of snails and the parasites being released from snails. Although there may be potential for indirect effects of atrazine on snails to have broad implications for disease dynamics (Daszak et al. 2001), current studies have left many questions that need to be addressed, including if there are direct effects of atrazine on snails.

Most studies assessing the effects of atrazine on freshwater snails have used different snail species (typically 1 species per study), different methods (including different scales), different endpoints, and different atrazine exposure concentrations, resulting in some conflicting results and limited general understanding. Additionally, despite the vast diversity of freshwater gastropods (Strong et al. 2008), interspecific variation in response to atrazine is rarely considered. Here, we address these concerns by (1) investigating acute and chronic effects of atrazine on four species of freshwater snails from three families (Planorbidae: *Biomphalaria glabrata, Helisoma trivolvis;* Physidae: *Physa acuta;* Lymnaeidae: *Stagnicola elodes*) using the same methods, endpoints, and concentrations, and (2) summarizing the current literature pertaining to the effects of atrazine on freshwater snails.

MATERIALS AND METHODS

Snails

Biomphalaria glabrata (Planorbidae) snails came from an inbred albino M-line, obtained from Sam Loker at the University of New Mexico. *Stagnicola elodes* were wild caught from Keith Co., NE in 2008 and the culture is also likely inbred. The remaining two species were wild caught [*Helisoma trivolvis* (Planorbidae): Stillwater, OK; *Physa acuta* (Physidae): Stillwater, OK] and reared in aquaria at Oklahoma State University for less than 1 yr. We chose these 4 species because they are common species used in toxicity assays, including atrazine, and represent 3 common and evolutionarily distinct freshwater snail families (Strong et al. 2008). *Biomphalaria glabrata* snails were originally identified by and sourced from the Biomedical Research Institute (Rockville, MD; http://www.afbr-bri.com/schistosomiasis). *Stagnicola elodes* and *Helisoma trivolvis* snails were identified using keys by Burch (1989) and Thorp and Covich (2009). *Physa acuta* snails were previously identified by penial morphology and mtDNA sequence data (Gustafson et al. 2014). Populations of the four snail species were maintained as previously described (see Bolek and Janovy 2007; Gustafson and Bolek 2015) in 10 gallon aquaria with under-gravel filters with a 12:12 hour light-dark cycle. Snails were fed a combination of fresh and frozen green leaf lettuce ad libitum supplemented with TetraMin tropical flakes fish food.

Materials

Atrazine (2-chloro-4-ethylamino-6-isopropylamino-s-triazine, 98% purity) was obtained from Chem Service, Inc. (West Chester, PA). All solvents were obtained from Fisher Scientific (Waltham, MA) and were pesticide grade or better. Dechlorinated water

was obtained by carbon filtration of Oklahoma State University tap water (pH: 7.4 – 7.9, Hardness: 80 – 100 mg calcium carbonate/L) and this water was used in all exposure tests and for maintenance of experimental cultures. Using gas-chromatography mass-spectrophotometry and select ion monitoring (see *Quality control* section for details), the dechlorinated water did not have detectable background levels of atrazine in water used directly for the test (limit of detection: <0.05 µg/L) and neither atrazine or metabolites desethylatrazine and deisopropylatrazine have been detected in this water source during regular monitoring conducted as part of ensuring culturing systems and running analytical blanks (limit of detection: <0.02 µg/L).

Acute atrazine exposure

Each speci es was exposed to a nominal 1,000 µg/L atrazine in dechlorinated tap water, using 123µL (0.000123%) of acetone as a carrier, for 48 hours in 1 L glass beakers with 10 randomly selected individuals per experimental unit. This experiment included three replicates of exposed and control (exposed to atrazine carrier only) beakers per species per treatment for a total of 24 beakers. Each beaker was covered with perforated Parafilm to ensure snails were in contact with water but could still breathe. Snails were not provided food during this time. Snails were classified as alive if they were moving or attached to the glass. Snails were classified as dead if they would not respond to prodding of the foot. One-way analyses of variance (ANOVA) with one-way Dunnett's post hoc tests were implemented in STATISTICA v10 (StatSoft, Inc., Tulsa, Oklahoma) to assess the acute effects of atrazine on snail survival.

Chronic atrazine exposure

Each snail species was tested during a separate experiment. For each experiment, a total of 320 unknown-age snails were haphazardly removed from aquaria and the size of each snail shell was measured to the nearest quarter mm using a handheld caliper. Because the two planorbid species (*B. glabrata* and *H. trivolvis*) have planispiral shells and most of the grown happens in width, size was measured as the width of the shell from outer lip of the aperture. For the conical-shelled species (*P. acuta* and *S. elodes*), size was measured as the length of the shell from the basal lip of the aperture to the apex. Snails were randomly placed into each of 32 glass bowls with 3L of dechlorinated tap water until each experimental unit contained 10 individuals. Snail size did not significantly differ between bowls for *B. glabrata* ($F_{31, 288} = 0.26$, $p = 0.99$), *H. trivolvis* ($F_{31, 288} = 0.65$, $p = 0.93$), *P. acuta* ($F_{31, 288} = 0.07$, $p = 0.99$), or *S. elodes* ($F_{31, 288} = 0.03$, $p = 0.99$). Bowls with snails were aerated and covered with fiberglass window screen to ensure snails could not escape but still allowed them to breathe. After a 1 wk acclimation period, bowls were randomly spiked with 1 of 4 treatment levels (solvent only, 0.3, 3, and 30 μg/L atrazine) resulting in 8 replicates per treatment. Addition of atrazine was carried out using 100 μL of acetone (0.00003%) which is not toxic to snails at low levels (0.0002%; Rohr et al. 2008a) and only mildly reduces snail fecundity at concentrations of 0.1% (Bluzat et al. 1979; Hutchinson et al. 2006). Snails were fed a combination of fresh and frozen green leaf lettuce ad libitum throughout the duration of the study. Water and relevant atrazine concentrations were replenished twice weekly (Monday and Friday) in each bowl for 28 d after the introduction of atrazine. During each water change, airline tubing, lettuce, and the glass bowls were visually examined for egg masses which could easily be

observed without magnification. All egg masses were counted and carefully removed with forceps. Egg masses were further examined under a dissecting microscope (Olympus SZ51) and all eggs within egg masses were counted. In addition, during the second water change of the week, the size of each snail was measured as previously described.

Within-bowl measurements included: the percent of snails that survived, average snail size, the average number of eggs masses laid per snail, and the average number of eggs per egg mass per snail. Because eggs were counted 3 or 4 d after a water change, the average number of eggs masses laid per snail and the average number of eggs per egg mass per snail were standardized by dividing the value by the number of days since the last water change. We used repeated measures (RM) ANOVAs to test the effects of atrazine on survival, growth, and reproduction. Statistics were conducted using STATISTICA v10 (StatSoft, Inc., Tulsa, Oklahoma).

Quality control

Water quality (i.e., ammonia, DO, water temperature) and atrazine concentrations were not measured for acute toxicity assays. However, water quality was measured for the chronic exposure study. Twice after the longest period between water changes (i.e., 4 d), water temperature, and percent dissolved oxygen were randomly subsampled from 4 bowls at each concentration for each chronic exposure study using a YSI DO Professional Series Probe (Yellow Springs, OH). In the same manner, ammonia (NH_3-N mg/L) was randomly subsampled once from 3 bowls at each concentration for each chronic exposure study using HACH Test 'N Tube kits (Nessler method 8038) and analyzed on a HACH UV spectrophotometer (DR 5000; Loveland, CO). To verify

nominal atrazine concentrations, 3 bowls at each concentration were randomly sampled once after the longest period between water changes (i.e., 4 d) for each chronic exposure study. To quantify atrazine in water, an internal standard of 100ng of atrazine-D_5 (Sigma Aldrich, St. Louis, MO) was added to each sample. Atrazine was extracted from water using C_{18} 6mL solid phase extraction cartridges (SampliQ, Agilent Technologies, Santa Clara, CA) that were previously conditioned with 5mL methanol. Atrazine was then eluted with 8mL ethyl acetate, and evaporated to 1mL using nitrogen gas. Samples were analyzed on a gas chromatograph-mass spectrometer (Agilent 5975c; Santa Clara, CA) using internal calibration and select ion monitoring (atrazine:200; atrazine- D_5:205).

Qualitative literature summary

We summarized all known literature pertaining to the effects of atrazine on freshwater snails. We searched Web of Science (Thomson Reuters, New York, NY) and Google Scholar with the terms "atrazine" combined with either "snail" or "gastropod." The citations from each study were also searched. Literature were summarized based on the biological level of organization tested, including subcellular, cellular, individual/population (sublethal or lethal), and community endpoints. We define community effects as those that affect snails via species interactions (e.g., consumer-resource, host-parasite). Data summaries include the observed effect, snail taxonomy, type of experiment, duration of experiment, number of replicates used, range of atrazine concentrations used, and the lowest observed effect concentration (LOEC). We only report LOECs when the study reports significant differences from controls. If a single study tested atrazine effects on endpoints from different levels of biological organization, each experiment was summarized independently. Studies using mixtures of atrazine with

an additional chemical were not included. All studies used technical grade atrazine; none used atrazine formulation. Numerous experiments, including those assessing sublethal effects, report no effect of atrazine on snail survival, thus, only studies testing survival above the lowest observed lethal concentration (i.e., 100 µg/L) were included in the survival category. To better identify the concentrations that exhibit biological effects on snails, each LOEC at each level of biological organization is graphically represented. Because of exponential differences in concentrations tested between levels of biological organization, statistics were not computed between categories.

RESULTS

Acute atrazine exposure

A nominal atrazine concentration of 1,000 µg/L did not significantly affect the survival of *H. trivolvis* (Dunnett's $p = 0.5$), *P. acuta* (Dunnett's $p = 0.5$), or *S. elodes* (Dunnett's $p = 0.81$) during the 48 hour exposure period. Statistics could not be computed (nor was it necessary) for *B. glabrata* because there was 100% survival in each experimental unit. In total, only 2 of 120 control snails died (1 *H. trivolvis* and 1 *P. acuta*). Similarly, only 3 of 120 exposed snails died (1 *H. trivolvis*, 1 *P. acuta*, and 1 *S. elodes*).

Chronic atrazine exposures

During the 28 day chronic exposure, atrazine concentrations were within 5.19 ± 3.68% (mean ± SD) of nominal concentrations and did not significantly differ between species within concentrations ($F_{6,24} = 1.08$, $p = 0.40$). In control treatments (i.e., solvent only), atrazine concentrations were below quantitation limit (<0.05 µg/L). Refer to the

Appendix A2.1 (supplementary table) for measured concentrations at each nominal concentration for each species. Water temperature (mean ± SD = 25.11 ± 0.75; min-max = 22.4-26.2 C), percent dissolved oxygen (91.3 ± 3.05; 83-96.4%), and ammonia concentrations (0.15 ± 0.08; 0.02-0.32 mg/L NH_3-N) remained consistent between species and between atrazine concentrations. Refer to Appendix A2. 1 (supplementary table) for water quality data at each nominal concentration for each species. Additionally, ammonia never reached toxic concentrations (i.e., >1mg/L: Alonso and Camargo 2003; Arthur et al. 1987).

Initial survival was high for all species with greater than 90% average survival among treatments across all species for the first 3 days (Fig. 2.1). Over the course of four weeks, survival significantly decreased with time for *B. glabrata*, *H. trivolvis*, and *S. elodes*; however, survival was not significantly affected by atrazine for any species (Table 2.1). *Physa acuta* had exceptionally high survival throughout the experiment and neither atrazine, time, or their interaction significantly affected survival (Table 2.1). By the end of the four weeks, *P. acuta* had the highest survival (mean across treatments ± SD: 94.1 ± 4.1%), followed by *S. elodes* (87.9 ± 4.2%), and *H. trivolvis* (77.5 ± 2.0%). *Biomphalaria glabrata* had the lowest end survival of the four species and exhibited the largest variation within and among treatments (61.5 ± 8.8%).

Over the course of 28 days, all species grew significantly, whereas neither atrazine nor the interaction had a significant effect on the size of *B. glabrata*, *H. trivolvis*, *P. acuta*, or *S. elodes* (Fig. 2.2; Table 2.1). *Stagnicola elodes* had the largest average increase in growth throughout the experiment going from an initial average length of 5.2 mm to an end length of 12.2 mm (Δ: 7.0 mm). Similarly, *Physa acuta* exhibited

asymptotic growth with an initial average length of 3.7 mm and an end length of 6.3 mm (Δ: 2.6 mm). In contrast, *B. glabrata* (initial: 13.9 mm; end: 14.6 mm; Δ: 0.7 mm) and *H. trivolvis* (initial: 11.5 mm; end: 12.5 mm; Δ: 1.0 mm) exhibited linear growth and a smaller (but significant) increase in width throughout the four weeks.

Time was also the only significant factor affecting the number of egg masses produced per snail per day for any species (Fig. 2.3; Table 2.1). *Physa acuta* was the only species that exhibited consistent egg laying patterns throughout the experiment. Although *B. glabrata* and *H. trivolvis* also produced egg masses throughout the experiment, there were several times when cumulative egg laying patterns plateaued (Fig. 2.3). The RM-ANOVA could not compute for *S. elodes* because of the consistent lack of egg production throughout the experiment (Fig. 2.3; Table 2.1).

Similarly, time was the only significant factor affecting the number of eggs produced per egg mass per snail per day for *B. glabrata*, *H. trivolvis*, and *P. acuta* (Fig. 2.4; time and atrazine component not presented because they are nearly identical to Fig. 2.3 and not significant). Again, the RM-ANOVA could not be computed for *S. elodes* because of the lack of egg production by this species throughout the experiment. There were significant differences between the average number of eggs produced per egg mass between species ($F_{3,3891} = 76.30$, $p < 0.001$) where *B. glabrata* produced more eggs per egg mass than *H. trivolvis* (Tukey's unequal N HSD $p < 0.001$) and *P. acuta* (Tukey's unequal N HSD $p < 0.001$; Fig. 2.4). Similarly, *S. elodes* produced more eggs per egg mass than *P. acuta* (Tukey's unequal N HSD $p < 0.001$; Fig. 2.4). All other pairwise comparisons were not significant (Tukey's unequal N HSD $p > 0.05$). Refer to the

Appendix A2.2 (supplementary table) for the performance of control snail survival, growth and reproduction.

Qualitative literature summary

The results of 54 experiments from 20 publications (including this study) are summarized in Table 2.2. Overall, atrazine effects have been tested on 11 freshwater snail species from 5 families. Of the 54 experiments, 39 (61.1%) used environmentally relevant atrazine concentrations (i.e., less than or equal to 100 μg/L). Similarly, 39 (61.1%) experiments used concentrations above 100 μg/L. Twelve experiments used a combination of concentrations that spanned from within, to above, ecologically relevant.

Relatively few experiments have been conducted at the subcellular level, and none for physid snails (Fig. 2.5). Nonetheless, significant effects are detected at the subcellular level within, and above, environmentally relevant concentrations. All but a single study reported an LOEC at the lowest concentration tested. Thus, subcellular LOECs should be considered upwardly biased. Similarly, cellular effects are commonly detected across common freshwater snail families within, and above, environmentally relevant concentrations (Table 2.2, Fig. 2.5). At the cellular level, the lowest reported LOEC was 10 μg/L which was also the lowest concentration tested. Reported LOECs for cellular effects should also be considered upwardly biased. Although few behavioral assays have been conducted (none for lymnaeids), atrazine-induced behavior alteration is also detected within, and above, environmentally relevant concentrations. Similar to cellular effects, the lowest reported LOEC for behavioral assays was 10 μg/L which was also the lowest concentration tested. Again, behavior LOECs should be considered upwardly biased. In contrast, and despite the broad range of snail species and atrazine

concentrations tested, most experiments observed no effect of environmentally relevant concentrations on sublethal or lethal endpoints with the lowest LOEC reported at 100 μg/L (Fig. 2.5; Table 2.2). Only studies above the lowest LOEC (i.e., 100 μg/L) were included in the survival category. Thus, reported survival LOECs should not be considered upwardly biased. Although few experiments have been conducted at the community level, significant indirect or interactive effects of atrazine on physid, planorbid, and lymnaeid species are frequently detected at, or near, environmentally relevant concentrations (Fig. 2.5). Similarly to subcellular, cellular, and behavioral assays, the majority of community-level LOECs were at the lowest concentration tested and may be upwardly biased.

DISCUSSION

Our qualitative literature review indicates that snails are often negatively affected by atrazine concentrations within, and exceeding, environmental relevance at low levels of biological organization, including the cellular and subcellular levels. However, effects on low levels of biological organization are typically not transitive to effects on the health of individuals or populations, including measure of growth, reproduction, and/or survival. It is important to keep in mind that most studies test atrazine in the absence of other stressors and do not reflect natural conditions where snails experience multiple stressors simultaneously. That being said, our acute exposures support this general trend and demonstrate that a high atrazine concentration (1,000 μg/L), above what typically occurs in nature, did not directly affect the survival of any of the four snail species we tested. Similarly, chronic exposures of environmentally relevant atrazine concentrations (0.3, 3,

30 μg/L) did not significantly affect growth, reproduction, or survival of any of the four snail species we tested. Although, in our study, the direct effects of environmentally relevant atrazine concentrations were not exhibited on population-level endpoints, our review indicates that atrazine can exhibit significant effects at the community level for some of these snail species (see Table 2.2). These studies suggest that environmentally relevant atrazine concentrations can indirectly or interactively affect freshwater snail reproduction, survival, abundance, and biomass by affecting consumer-resource or host-parasite relationships. Because each experiment within each biological level of organization used different endpoints, concentrations, durations, and species, we discuss each study, including ours, in more detail below.

Although subcellular effects of atrazine were detected in five studies on three species of freshwater snails, some of these studies used unrealistic exposure concentrations. Baturo et al (1995) found no effect of environmentally relevant concentrations of atrazine (5-125 μg/L) on the functionality of enzymes responsible for glycogen utilization in *L. palustris*. However, a follow up study revealed a significant 30% inhibition of detoxification enzymes (benzo[*a*]pyrene hydroxylase and glutathione *S*-transferase) at the same concentrations (Baturo and Lagadic 1996). At a much higher atrazine concentration (330 μg/L), Barky et al. (2012) observed reduced activities (max 79%) of several enzymes in *Biomphalaria alexandrina*, including glycogen phosphorylase, superoxide dismutase, catalase, glutathion reductase, succinin dehydrogenase, acetylcholinesterase, lactic dehydrogenase, and two phosphatases. In contrast, they found increased activity (max 53%) of lipid peroxide and transaminases. At an environmentally unrealistic atrazine concentration of 10,100 μg/L, Omran and Salama

(2013) observed a significant 50% reduction in steroid levels (testosterone and 17β-estradiol) in *B. alexandrina* snails; however, they also observed a significant 50% increase in cytochrome P450B1-like immunoreactivity at the same concentration. At similar concentrations (5,000 – 10,000 µg/L), Mona et al. (2013) used isozyme (α- and β-esterase, peroxidase, and catalase) assays and reported a significant 44% incrase in genotoxicity.

All but one of the eleven studies assessing atrazine toxicity at the cellular level reported significant effects. Rosés et al. (1999) found atrazine induced kidney cell lysis in *P. acuta* at 100 µg/L, but not in *Ancylus fluvuatilis* up to 20,000 µg/L; they also found no toxicity to hepatopancreas cells in either species. Several studies have observed significantly higher levels of circulating haemocytes (up to 770%) in response to atrazine concentrations ranging from 10 - 24,000 µg/L (Russo and Lagadic 2000; Russo and Lagadic 2004; Sandland and Carmosini 2006). Similarly, Russo and Lagadic (2004) and Russo et al. (2009) observed a significant 60% decrease in phagocytic activity (50 µg/L atrazine) and a significant 330% increase in lysosomal fragility (10 µg/L atrazine) in *Lymnaea stagnalis*, respectively. Taken together, these studies suggest atrazine negatively affects snail immunity. Sawasdee and Köhler (2009) observed a significant 10% reduction (100 µg/L) of embryo hatching in *Marisa conuarietis* but no effect on eye or tentacle development (up to 30,000 µg/L atrazine). In addition to observations on the negative effects of embryo hatching, Barky et al. (2012) observed an 85% increase in egg abnormalities in *B. glabrata* at 330 µg/L atrazine. Finally, Omran and Salama (2013) observed an increase in azoospermia (i.e., lack of sperm) and oocyte deformations for *B.alexandrina* at 10,100 µg/L atrazine, possibly as a result of altered steroid levels.

Although atrazine effects on the subcellular and cellular levels were observed at, or above, environmentally relevant atrazine concentrations, few of these studies have found sublethal effects on individual snails of these species over a similar range of concentrations.

Of the four studies testing the effects of atrazine on growth at, or near, ecologically relevant concentrations (ranging from 5-201 μg/L), none found significant effects (Baturo and Lagadic 1996; Baturo et al. 1995; Gerard and Poullain 2005; Rohr et al. 2008a). Our chronic toxicity tests also showed no direct effects of atrazine (up to 30 μg/L) on growth of *B. glabrata*, *H. trivolvis*, *P. acuta*, or *S. elodes*. *Physa acuta* and *S. elodes* exhibited asymptotic growth curves whereas *B. glabrata* and *H. trivolvis* exhibited small (but significant) linear increases in size over the 28 days. Although we have no reason to expect significant atrazine effects on growth (based on other studies), we may not have been able to adequately detect atrazine effects on these two planorbid species because of their relatively large initial sizes and slow linear growth. This is partly an artefact of the population dynamics within our culture tanks. Although beginning toxicity assays at a smaller size would likely have resulted in relatively more growth, these two planorbid species generally exhibit slow linear growth throughout their entire lives (Boerger 1975; Lévêque and Pointier 1976) and may simply require more time to grow in order to observe any cumulative effects on growth.

Of the studies testing the effects of atrazine on snail reproduction, most observed no significant effects (Baturo and Lagadic 1996; Baturo et al. 1995; Baxter et al. 2011; Gerard and Poullain 2005; Muñoz et al. 2001; Rohr et al. 2008b). Similarly, our chronic toxicity tests showed no direct effects of atrazine (up to 30 μg/L) on the reproduction of

B. glabrata, *H. trivolvis*, *P. acuta*, or *S. elodes*. However, *S. elodes* produced very few eggs and we were unable to realistically test for reproductive effects for this species. Interestingly, Baxter et al. (2011) also used *S. elodes* snails in their study and report low variation in egg production (often no egg production) within their microcosms. Similar to our study, the conditions of Baxter et al. (2011) may not have been conducive for egg production in *S. elodes*. Until better techniques are developed that promote reproduction in some lymnaeid snails within a toxicity testing system, *S. elodes* is not a useful species for reproductive toxicity assays. In contrast, *B. glabrata*, *H. trivolvis*, and *P. acuta* in particular, are suitable models for future reproductive toxicity assays.

In contrast to studies mentioned above, Streit and Peter (1978) found negative effects of atrazine (40% reducation) at 4,000 and 16,000 µg/L on *A. fluviatilis* reproduction over 40 days but did not report any statistics. Additionally, Sandland and Carmosini (2006) observed a 25% reduction in reproduction in *Physa gyrina* but only after 8 weeks of exposure to 100 µg/L atrazine. This could have been the result of an interactive effect of atrazine with age because *Physa* snails typically only live for a few months and exhibit multiple generations per growing season (Brown 1979; Clampitt 1970). Similarly, Barky et al. (2012) exposed *B. alexandrina* to 330 µg/L atrazine for 6 weeks and observed significantly reduced egg production (complete inhibition of reproduction after 3 weeks). These studies demonstrate that sublethal effects on growth and reproduction, although rare, can occur at concentrations above what is found in nature. However, this is not to say atrazine does not have important implications for individual snails because, as our review indicates, atrazine has been shown to mediate snail behavior by either increasing (up to 300%) (Rosés et al. 1999; Streit and Peter 1978)

or decreasing (down to 19%) (Gerard and Poullain 2005) snail locomotion or grazing behavior.

Although several studies have observed reduced survival of freshwater snails in response to atrazine, most have been at exceedingly high concentrations (Barky et al. 2012; Omran and Salama 2013; Russo and Lagadic 2000; Streit and Peter 1978). An additional study found the survival of *Physa gyrina* was reduced by 25% at an environmentally relevant atrazine concentration of 100 µg/L but only after an 8 week exposure to a relatively short lived species (Sandland and Carmosini 2006). Our acute (1,000 µg/L for 48 h) and chronic (up to 30 µg/L for 28 d) toxicity tests showed no direct effects of atrazine on the survival of *B. glabrata*, *H. trivolvis*, *P. acuta*, or *S. elodes*. Interestingly, the two planorbid species (*B. glabrata* and *H. trivolvis*) exhibited the lowest survival of the four species. As previously noted, both species exhibited relatively large initial sizes and slow linear growth which indicates they were mature adults. Thus, the relatively lower mean survival and relatively higher variability in survival may simply be a result of age. Nonetheless, the lack of atrazine effects on survival is consistent with other studies. Rosés et al. (1999) exposed two species (*P. acuta* and *A. fluviatilis*) to 2,000, 10,000, and 20,000 µg/L atrazine and also found no effect on survival after 48 hours. Sawasdee and Köhler (2009) exposed *M. conuarietis* snails up to 30,000 µg/L atrazine for 14 days and also observed no effects. Additionally, Rohr et al. (2008a) exposed *Planorbella trivolvis* (= *H. trivolvis*) snails to 201 µg/L for 14 days and found no significant differences relative to controls. Interestingly, lethal concentration values have been reported from three studies. Russo and Lagadic (2000) exposed *L. palustris* snails to 24,000 µg/L atrazine for 96 hours and report an LC_{20} (the concentration that was

lethal to 20% of the individuals) at that concentration. Barky et al. (2012) reported an LC_{90}, LC_{50}, and LC_{10} for *B. alexandrina* at 4,750, 1,250, and 330 µg/L atrazine, respectively. Additionally, Omran and Salama (2013) report an LC_{50} and LC_{10} for *B. alexandrina* at 101,160 and 10,100 µg/L atrazine, respectively. Although these reported lethal concentrations are higher than concentrations found in nature, none of the studies report whether survival differences were significantly different from controls and as a result, it is difficult to interpret the biological meaning of these findings. Combined, these studies suggest that atrazine-induced mortality only occurs at extremely high and environmentally unrealistic concentrations or after several months of continuous exposure at concentrations above what is typically detected in nature.

Interestingly, even though direct effects of atrazine on snail populations may be improbable, interactive and indirect effects have been reported at environmentally relevant concentrations. For example, Sandland and Carmosini (2006) found the interaction of predation cues and atrazine significantly reduced reproduction in *P. gyrina* by 25% while significantly increasing circulating haemocytes by 100%. Koprivnikar and Walker (2011) observed a significant 40% reduction in survival of *S. elodes* snails simultaneously exposed to 0.33 µg/L desethylatrazine (an atrazine metabolite) and naturally infected with a gynmocephalus-type digenetic trematode species. Rohr and Crumrine (2005) observed significant indirect effects of atrazine (25 µg/L) which reduced *H. trivolvis* snail mass by 7% and reproduction by 15% via increasing resource competition with other species. Rohr et al. (2008b) later observed a 4-fold increase in *H. trivolvis* snail abundance as a result of atrazine (117 µg/L) inhibiting the growth of phytoplankton and indirectly increasing the biomass of periphyton, which is a primary

food source of some freshwater snails. It is unclear if this pattern would be similar for different snail species because of species specific differences in feeding preferences (Thorp and Covich 2009). For example, Brown (1982) demonstrated different food preferences between physid, planorbid, and lymnaeid snails sourced from the same locations, where *L. elodes* preferred carrion and periphyton, and *P. gyrina* and *H. trivolvis* were generalists with low positive or negative food preferences.

In contrast to the study by Rohr et al. (2008b), Baxter et al. (2011) found no effects of atrazine (up to 100 µg/L) on phytoplankton biomass, periphyton biomass, *S. elodes* snail abundance, or on abundance when pooling data from multiple *Physella* (=*Physa*) species. Further research by Baxter et al. (2013) indicates that phosphorous, even in the presence of atrazine, can cause significant changes in primary production (similar to Johnson et al. 2007). Baxter et al. (2013) suggest the apparent effects of atrazine observed by Rohr et al. (2008b) may be driven by phosphorous which is often correlated with atrazine. Currently, there is no consensus on this topic (Baxter et al. 2012; Rohr et al. 2012). Nonetheless, these studies demonstrate that indirect effects of atrazine are not easily predictable. For example, atrazine effects on primary production could result in changes in snail abundance, biomass, or reproduction. Alternatively, atrazine could affect other aquatic organisms which could increase or decrease competitive interactions. The interactive and indirect effects of atrazine and other pesticides on aquatic communities and snail communities should be explored further.

Finally, atrazine is transformed in the environment to several relatively environmentally-stable metabolites, including desethylatrazine and deisopropylatrazine, which are commonly found in surface waters (Graymore et al. 2001; Solomon et

al.1996). Despite the common occurrence of these metabolites, their direct effects on snails have not been thoroughly tested. For example, only a single study has addressed the effects of an atrazine metabolite on snail health and found no direct effects of a single low concentration (0.33 μg/L) of desethylatrazine on snail survival (Koprivnikar and Walker 2011). In the current study, concentrations of atrazine were stable, suggesting limited degradation in water and thus very low concentrations of metabolites in the test system, which was confirmed in preliminary studies to be below detection limits of the method (<0.05 μg/L). In short-term laboratory studies, it is not likely that metabolites were formed. However, in longer-term mesocosm studies, metabolite formation may have occurred and the toxicological response should be considered as a response to atrazine and metabolites.

Conclusions

Studies indicate that atrazine affects snails from the subcellular to the community level and atrazine-snail responses appear to be similar among major freshwater snail families. At lower biological levels, environmentally relevant atrazine concentrations can affect enzyme activity, immunity, can cause cell and tissue damage, and can modify snail behavior in the absence of other stressors. However, many studies, including ours, suggest suborganismal effects rarely translate into individual or population level effects such as growth, reproduction, or survival. This is particularly true for environmentally relevant atrazine concentrations which did not elicit life history effects on a broad range of species in the absence of other stressors. However, it is important to keep in mind that although the effects on natural populations may not occur directly through atrazine as an active chemical, but through other compounds that are inherently associated with their

presence in the field (e.g., solvents, surfactant), or with additional stressors. Interestingly, some studies suggest that atrazine effects reappear at the aquatic community level and indirectly or interactively affect snail reproduction, survival, and biomass at environmentally relevant concentrations. Given the importance of snails in nutrient cycling, food webs, and parasite transmission, indirect effects of atrazine, and other pesticides, have the broadest implications for downstream or cascading effects on aquatic ecosystem dynamics and parasite transmission.

It is important to keep in mind that there are approximately 4,000 species of freshwater gastropods from 33 families (Strong et al. 2008) and only 11 species from 5 families have been evaluated thus far. Future studies should include a variety of different species, use ecologically relevant concentrations, and consider the usefulness of each species for specific toxicity assays. For example, our data suggest *P. acuta* and *S. elodes* are useful species for growth assays whereas *B. glabrata*, *H. trivolvis*, and particularly *P. acuta* are useful for reproductive assays. One potential avenue for future study would be to assess the relationship between chemically-induced changes in immune function (i.e., haemocytes) and parasite (i.e., trematode) transmission. Additionally, indirect effects of chemicals on consumer-resource relationships should be explored further considering snails as the consumer and also as the resource.

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Table 2.1. Summary of RM-ANOVAs testing the effects of atrazine on survival, growth, egg masses produced per snail per day, and eggs produced per egg mass per snail per day. Degrees of freedom (treatment, error) are reported with *F* statistic. Two RM-ANOVAs could not compute (--) for *Stagnicola elodes* because of the lack of reproduction throughout the experiment.

Species	Survival		Growth		Egg Masses		Eggs	
	F	*p*	*F*	*p*	*F*	*p*	*F*	*p*
Biomphalaria glabrata								
atrazine	3,25 = 1.15	0.35	3,25 = 0.39	0.76	3,25 = 0.22	0.88	3,25 = 0.44	0.73
time	7,175 = 25.72	<0.001	4,100 = 16.09	<0.001	7,175 = 16.09	<0.001	7,175 = 4.33	<0.001
atrazine*time	21,175 = 0.60	0.92	12,100 = 0.82	0.63	21,175 = 0.42	0.99	21,175 = 0.78	0.74
Helisoma trivolvis								
atrazine	3,28 = 0.15	0.93	3,26 = 1.61	0.21	3,26 = 1.38	0.27	3,26 = 2.51	0.08
time	7,196 = 14.10	<0.001	4,104 = 35.84	<0.001	7,182 = 56.92	<0.001	7,182 = 12.16	<0.001
atrazine*time	21,196 = 0.24	0.99	12,104 = 1.60	0.10	21,182 = 1.35	0.15	21,182 = 0.90	0.59
Physa acuta								
atrazine	3,28 = 1.26	0.31	3,28 = 1.80	0.17	3,28 = 1.35	0.28	3,28 = 1.27	0.30
time	7,196 = 0.92	0.56	4,112 = 507.23	<0.001	7,196 = 14.74	<0.001	7,196 = 5.83	<0.001
atrazine*time	21,196 = 0.92	0.56	12,112 = 1.43	0.16	21,196 = 1.47	0.09	21,196 = 1.41	0.12
Stagnicola elodes								
atrazine	3,28 = 1.16	0.34	3,26 = 0.50	0.69	--	--	--	--
time	7,196 = 9.95	<0.001	4,104 = 436.59	<0.001	--	--	--	--
atrazine*time	21,196 = 1.23	0.22	12,104 = 0.76	0.69	--	--	--	--

Table 2.2. Summary of studies testing atrazine toxicity to freshwater snails including the effect level and endpoint, snail species, type of experiment (lab, micro/mesocosm [cosm]), duration of the experiment (days), number of replicates per concentration (replicates are reported as experimental unites; if statistics were conducted on individual snails the sample size per treatment is in parentheses; NR: not reported), range of concentrations tested (Range), and lowest observed effect concentration (LOEC). Table is sorted alphabetically by snail family then snail species. Only studies specifically testing for survival at or above 100 µg/L (lowest LOEC) were included in the survival category.

Effect Level[a]	Snail		Experiment			Atrazine conc. (µg/L)			Reference
Magnitude of effect (%) on endpoint	Family	Species	Type	Duration	Replicates	Measured	Range	LOEC[b]	
Subcellular									
NE on glucose enzymes	Lymnaeidae	*Lymnaea palustris*	Cosm	25	3	No	5-125	>125	Baturo et al. 1995
↓30 detoxification enzymes	Lymnaeidae	*Lymnaea palustris*	Cosm	21	3	Yes	5-125	5	Baturo and Lagadic 1996
↓79↑53 enzymes, ↓50 DNA synthesis	Planorbidae	*Biomphalaria alexandrina*	Lab	42	(10)	No	330	330	Barky et al. 2012
↓50 steroids, ↓50 proteins, ↑50 CYP450	Planorbidae	*Biomphalaria alexandrina*	Lab	21	(50)	No	10,100	10,100	Omran and Salama 2013
↑44 genotoxicity	Planorbidae	*Biomphalaria glabrata*	Lab	21	(15)	No	5,000-10,000	5,000	Mona et al. 2013
Cellular									
↓10 embryos, NE on development	Ampullariidae	*Marisa conuarietis*	Lab	10	9	No	100-30,000	100	Sawasdee and Köhler 2009
↑770 haemocytes	Lymnaeidae	*Lymnaea palustris*	Lab	28	(3-8)	No	24,000	24,000	Russo and Lagadic 2000
↑160 haemocytes	Lymnaeidae	*Lymnaea stagnalis*	Lab	21	(10)	No	10-100	10	Russo and Lagadic 2004
↓60 phagocytic activity	Lymnaeidae	*Lymnaea stagnalis*	Lab	21	(4-10)	No	10-100	50	Russo and Lagadic 2004
↑330 lysosomal fragility	Lymnaeidae	*Lymnaea stagnalis*	Lab	21	4	Yes	10-101	10	Russo et al. 2009
↑Q kidney cell lysis	Physidae	*Physa acuta*	Lab	10	(15)	No	20-20,000	100	Rosés et al. 1999
↑300 haemocytes	Physidae	*Physa gyrina*	Lab	56	(5)	No	100	100	Sandland and Carmosini 2006
↓60 embryo hatching	Planorbidae	*Ancylus fluviatilis*	Lab	45	(20)	Yes	1,000-16,000	1,000[c]	Streit and Peter 1978
NE on kidney/hepatopancreas cells	Planorbidae	*Ancylus fluviatilis*	Lab	10	(15)	No	20-20,000	>20,000	Rosés et al. 1999
↑85 egg abnormalities, ↓60 hatching	Planorbidae	*Biomphalaria alexandrina*	Lab	42	(50)	No	330	330	Barky et al. 2012
↑Q azoospermia, ↓Q oocytes	Planorbidae	*Biomphalaria alexandrina*	Lab	21	(50)	No	10,100	10,100	Omran and Salama 2013
Individual/population - behavior									
↓19 locomotion	Heterobiidae	*Potamopyrgus antipodarum*	Lab	35	(90-180)	No	10-50	10	Gerard and Poullain 2005
NE on escaping	Physidae	*Physa acuta*	Lab	18	3	Yes	14	>14	Muñoz et al. 2001

↑45 velocity	Physidae	*Physa acuta*	Lab	18	(15)	No	15	15	Rosés et al. 1999
↑70 velocity	Planorbidae	*Ancylus fluviatilis*	Lab	18	(15)	No	15	15	Rosés et al. 1999
↑300 foraging	Planorbidae	*Ancylus fluviatilis*	Lab	40	(20)	Yes	1,000-16,000	1,000[c]	Streit and Peter 1978
Individual/population - sublethal									
NE on growth, reproduction	Heterobiidae	*Potamopyrgus antipodarum*	Lab	35	(90-180)	No	10-50	>50	Gerard and Poullain 2005
NE on growth	Lymnaeidae	*Lymnaea palustris*	Lab	70	3	No	5-125	>125	Baturo et al. 1995
NE on reproduction	Lymnaeidae	*Lymnaea palustris*	Lab	84	3	No	5-125	>125	Baturo et al. 1995
NE on growth, reproduction	Lymnaeidae	*Lymnaea palustris*	Cosm	21	3	Yes	5-125	>125	Baturo and Lagadic 1996
NE on reproduction	Lymnaeidae	*Stagnicola elodes*	Cosm	73	3	Yes	1-100	>100	Baxter et al. 2011
NE on growth, reproduction	Lymnaeidae	*Stagnicola elodes*	Lab	28	8	Yes	0.3-30	>30	This Study
NE on biomass, reproduction	Physidae	*Physa acuta*	Lab	18	3	Yes	14	>14	Muñoz et al. 2001
NE on growth, reproduction	Physidae	*Physa acuta*	Lab	28	8	Yes	0.3-30	>30	This Study
↓25 reproduction	Physidae	*Physa gyrina*	Lab	56	(24-27)	No	100	100	Sandland and Carmosini 2006
NE on reproduction	Physidae	*Physa spp.*	Cosm	73	3	Yes	1-100	>100	Baxter et al. 2011
↓40 reproduction	Planorbidae	*Ancylus fluviatilis*	Lab	40	(10)	Yes	1,000-16,000	4,000[c]	Streit and Peter 1978
↓100 reproduction	Planorbidae	*Biomphalaria alexandrina*	Lab	42	(50)	No	330	330	Barky et al. 2012
NE on growth, reproduction	Planorbidae	*Biomphalaria glabrata*	Lab	28	8	Yes	0.3-30	>30	This Study
NE on growth, reproduction	Planorbidae	*Helisoma trivolvis*	Lab	28	(20)	Yes	201	>201	Rohr et al. 2008a
NE on growth, reproduction	Planorbidae	*Helisoma trivolvis*	Lab	28	8	Yes	0.3-30	>30	This Study
Individual/population - survival									
NE on survival	Ampullariidae	*Marisa conuarietis*	Lab	14	9	No	100-30,000	>30,000	Sawasdee and Köhler 2009
NE on survival	Lymnaeidae	*Lymnaea palustris*	Cosm	84	3	No	5-125	>125	Baturo et al. 1995
↓20 survival	Lymnaeidae	*Lymnaeae palustris*	Lab	3	(50)	No	24,000	24,000[c]	Russo and Lagadic 2000
NE on survival	Lymnaeidae	*Stagnicola elodes*	Lab	2	3	No	1,000	>1,000	This Study
NE on survival	Physidae	*Physa acuta*	Lab	2	3	No	20-20,000	>20,000	Rosés et al. 1999
NE on survival	Physidae	*Physa acuta*	Lab	2	3	No	1,000	>1,000	This Study

↓25 survival	Physidae	*Physa gyrina*	Lab	56	(24-27)	No	100	100	Sandland and Carmosini 2006
↓40 survival, ↓Q biomass	Planorbidae	*Ancylus fluviatilis*	Lab	40	(20)	No	1,000-16,000	4,000[c]	Streit and Peter 1978
NE on survival	Planorbidae	*Ancylus fluviatilis*	Lab	2	3	No	20-20,000	>20,000	Rosés et al. 1999
↓10 survival	Planorbidae	*Biomphalaria alexandrina*	Lab	42	NR	No	330	330[c]	Barky et al. 2012
↓10 survival	Planorbidae	*Biomphalaria alexandrina*	Lab	21	(50)	No	10,100	10,100	Omran and Salama 2013
NE on survival	Planorbidae	*Biomphalaria glabrata*	Lab	2	8	No	1,000	>1,000	This Study
NE on survival	Planorbidae	*Helisoma trivolvis*	Lab	28	(20)	Yes	201	>201	Rohr et al. 2008a
NE on survival	Planorbidae	*Helisoma trivolvis*	Lab	2	8	No	1,000	>1,000	This Study
Interactive or indirect community effects									
NE on abundance	Lymnaeidae	*Stagnicola elodes*	Cosm	73	3	No	1-100	>100	Baxter et al. 2011
↓40 survival	Lymnaeidae	*Stagnicola elodes*	Lab	41	(15-22)	No	0.33 DE	0.33 DE	Koprivnikar and Walker 2011
↑100 haemocytes, ↓25 reproduction	Physidae	*Physa gyrina*	Lab	56	(24-27)	No	100	100	Sandland and Carmosini 2006
NEs on abundance	Physidae	*Physa spp.*	Cosm	73	3	Yes	1-100	>100	Baxter et al. 2011
↓7 snail mass, ↓15 reproduction	Planorbidae	*Helisoma trivolvis*	Cosm	28	4	No	25	25	Rohr and Crumrine 2005
↑400 abundance	Planorbidae	*Helisoma trivolvis*	Cosm	28	4-8	Yes	117	117	Rohr et al. 2008b

[a]NE: no effect; ↑: increased, inducted, or activated; ↓: decreased, reduced, or inhibited; Q: qualitative observations; for magnitude of effect, we report the most sensitive endpoint
[b]>: the LOEC is either greater than the values tested or the experiment did not have enough statistical power; DE: atrazine metabolite desethylatrazine
[c]Projected value; statistics were not reported

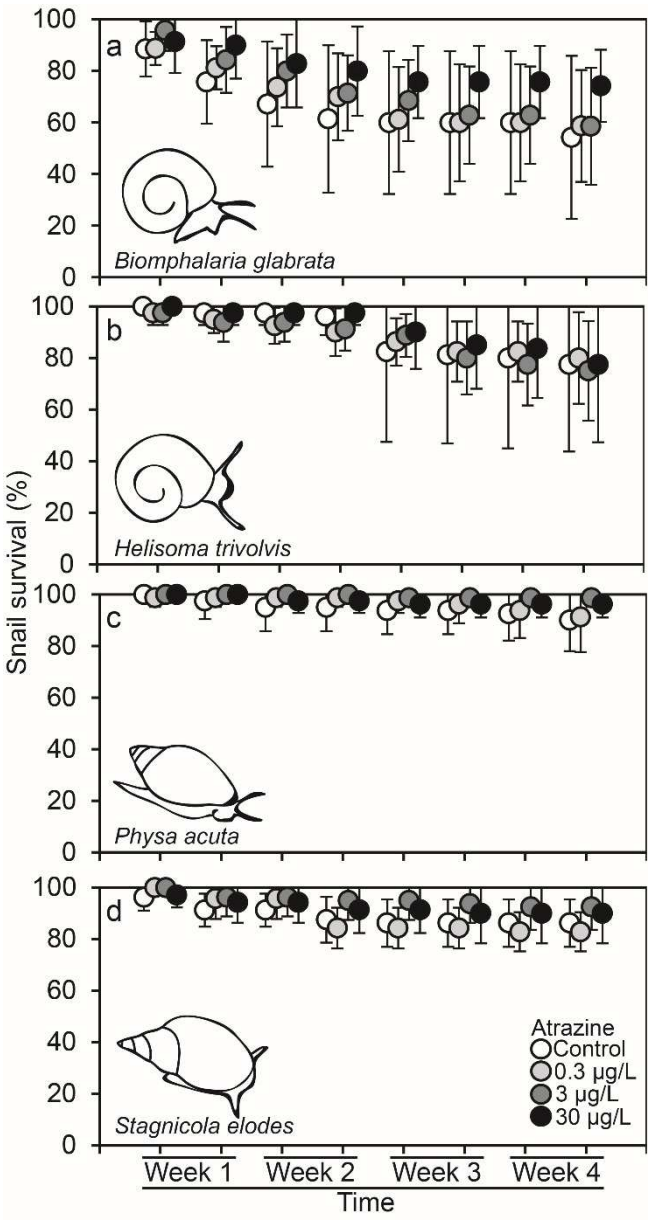

Fig. 2.1 Effects of atrazine on snail survival (mean ± SD) for (a) *Biomphalaria glabrata*, (b) *Helisoma trivolvis*, (c) *Physa acuta*, and (d) *Stagnicola elodes*. Means are calculated from the number of bowls (n=8) per concentration (n=4) for each species. Mean values are offset on the x-axis for visualization purposes only. Snail drawings are not to scale and are presented for visual reference.

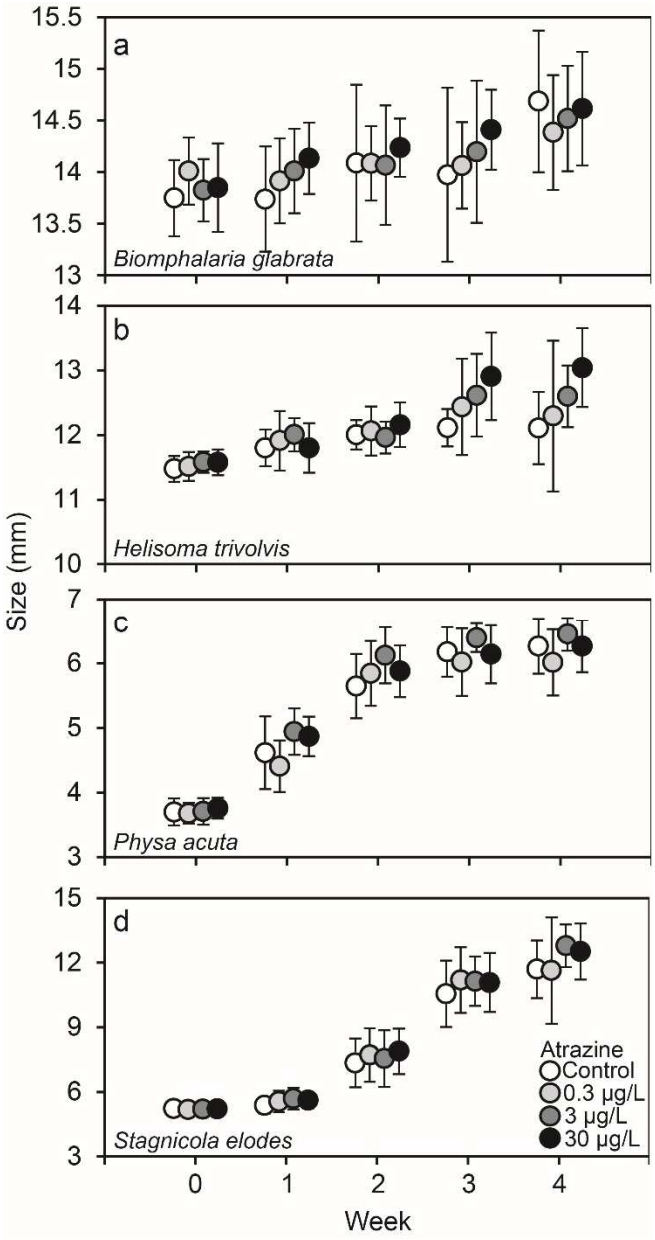

Fig. 2.2 Effects of atrazine on snail size (mean ± SD) for (a) *Biomphalaria glabrata*, (b) *Helisoma trivolvis*, (c) *Physa acuta*, and (d) *Stagnicola elodes*. Means are calculated from the number of bowls (n=8) per concentration (n=4) for each species. Mean values are offset on the x-axis for visualization purposes only.

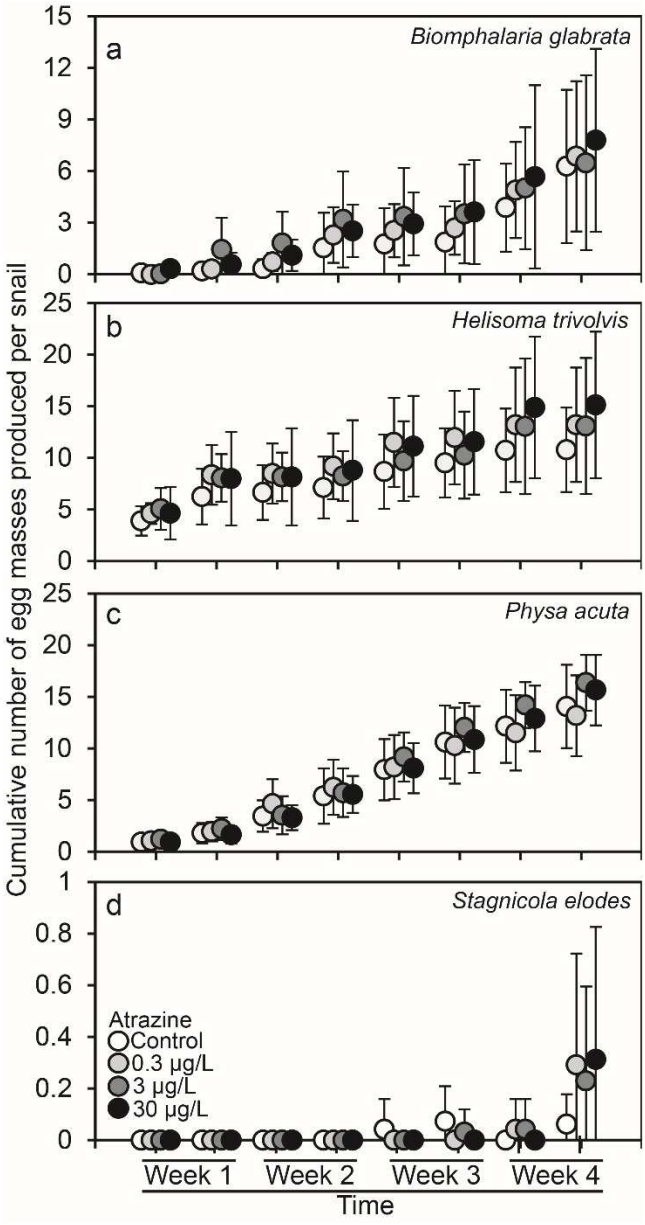

Fig. 2.3 Effects of atrazine on the number of egg masses produced per snail (mean ± SD) for (a) *Biomphalaria glabrata*, (b) *Helisoma trivolvis*, (c) *Physa acuta*, and (d) *Stagnicola elodes*. Means are calculated from the number of bowls (n=8) per concentration (n=4) for each species. Mean values are offset on the x-axis for visualization purposes only.

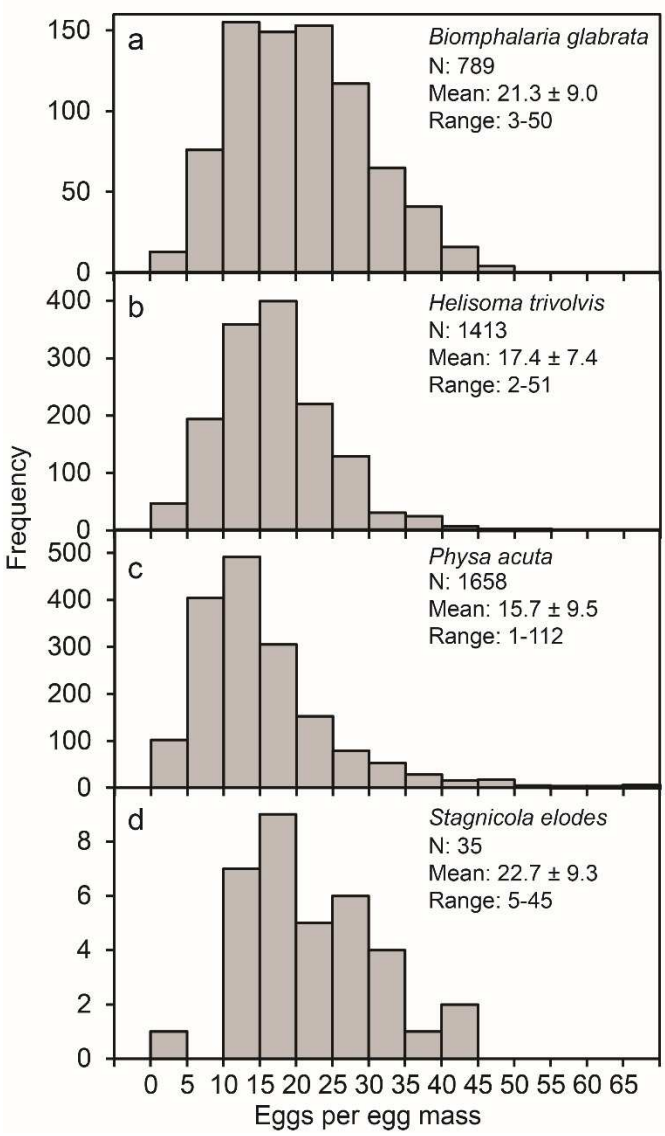

Fig. 2.4 Frequency distribution of eggs produced per egg mass for (a) *Biomphalaria glabrata*, (b) *Helisoma trivolvis*, (c) *Physa acuta*, and (d) *Stagnicola elodes*. Sample sizes, means ± SDs, and ranges are reported for each species and include all egg masses laid throughout the duration of each experiment. The last bin includes all values greater than or equal to 65.

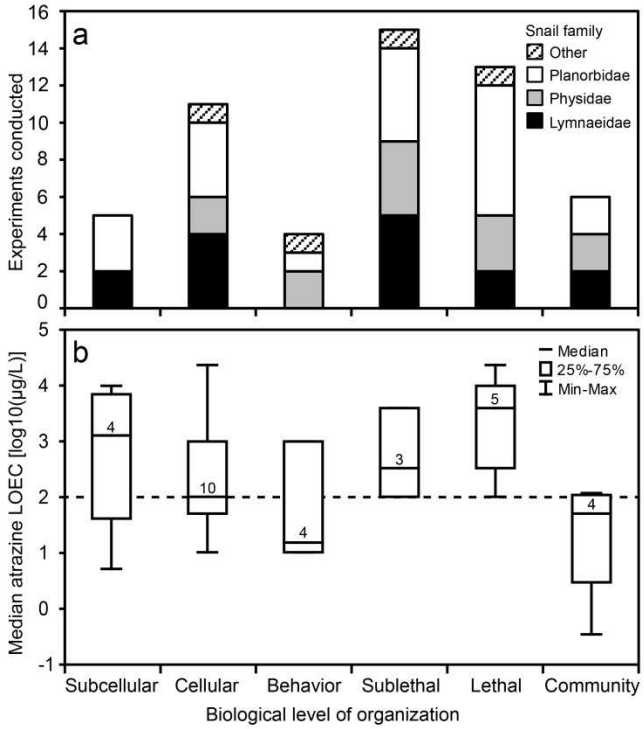

Fig. 2.5 Summary of studies testing atrazine toxicity to freshwater snails at different levels of biological organization including (a) the number of experiments conducted based on snail taxonomy, and (b) the median, 25-75% interquartiles, and the range of lowest observed effect concentrations (LOECs) in each category. All experiments reported in Table 2.2 are included in (a); however, only those with LOECs are included in (b); the sample size is indicated above the median. The dashed line indicates 100 µg/L; values below the line indicate environmentally relevant concentrations, whereas values above the line indicate values above those typically found in nature (Graymore et al. 2001).

CHAPTER III

TRADEOFF BETWEEN ESTABLISHING AN INFECTION AND KILLING THE HOST: RESPONSE OF SNAILS (*PHYSA ACUTA*) TO A GRADIENT OF TREMATODE (*HALIPEGUS ECCENTRICUS*) EXPOSURES

Gustafson KD, Bolek MG (2015) Tradeoff between establishing an infection and killing the host: Response of snails (*Physa acuta*) to a gradient of trematode (*Halipegus eccentricus*) exposures. Journal of Parasitology. 101:104-107. doi:10.1645/14-563.1

The research was original and primarily conducted under the advisement of my advisor, Matthew Bolek.

ABSTRACT

Pond snails (*Physa acuta*) were exposed to a gradient (0, 1, 3, 5, 10, 15, 20, 25, 30, 35, 40, and 50) of trematode (*Halipegus eccentricus*) eggs and observed to determine egg dose effects on the survival of snails through the prepatent period, snail reproduction, and the production of cercariae. The probability of snail survival through the prepatent period significantly decreased with increasing egg exposures, where the odds of snail survival was 0.94 per trematode egg. Similarly, the probability of snail reproduction significantly decreased with increasing egg exposures, where the odds of snail reproduction was 0.85 per trematode egg. In contrast, the probability of a snail shedding cercariae significantly increased with increasing egg exposures, where the odds of reaching a patent infection were 1.64 per trematode egg. However, snails shedding cercariae that were exposed to higher doses of trematode eggs tended to die sooner. Thus, there appears to be a tradeoff between infecting a snail and killing the host.

INTRODUCTION

In many snail-trematode systems, low infection intensities can be eliminated through snail immune responses whereas over-infections often result in parasite-induced mortality (Vanderknaap and Loker, 1990). Although there has been considerable interest in the effects of trematode infections on snail-trematode life-history characteristics (Minchella, 1985; Sorensen and Minchella, 2001), most studies have treated the infection as successful or unsuccessful (i.e., a binary effect). As a result, few studies have considered trematode dose-dependent effects on snail hosts or on the establishment of an infection within gastropods (but see Makanga, 1981; Gérard et al., 1993; Zakikhani and

Rau, 1998). One major reason these studies are rare may simply be because of the lack of domesticated trematode systems to test such relationships. Additionally, many of the life cycles that have been domesticated (e.g., schistosomes and echinostomes) use free-swimming miracidia to infect snail hosts (Shoop, 1988) which makes laboratory exposures more difficult to control compared to trematode life cycles where snails ingest eggs with fully developed miracidia.

Recently, the lifecycle of *Halipegus eccentricus* has been domesticated in the laboratory (Bolek et al., 2010). *Halipegus eccentricus* is site specific to the eustachian tubes of true frogs and infects physid snails as the first intermediate host (Bolek et al., 2010). Unlike many digenean trematodes that have ciliated, free-swimming miracidia stages (Shoop, 1988), the miracidia of *Halipegus* spp. are unciliated and do not hatch outside of a snail host; thus, snails must ingest embryonated eggs with developed miracidia to become infected (Thomas, 1939). Given that first-intermediate physid snail hosts are easy to culture (Bolek and Janovy, 2007), and the relatively large filamentous eggs of *H. eccentricus* are easy to manipulate, *H. eccentricus* is a useful system for testing dose-dependent effects on snail host health and for studying factors affecting trematode life cycles in general.

Previously, Zelmer and Esch (2000) tested the effects of *H. occidualis* egg dose on cercarial production in *Helisoma anceps* snails; however, they did not test the dosage effects on snail life history characteristics. Additionally, no study has quantified the number of *H. eccentricus* eggs an individual *Physa* spp. snail must ingest to become infected and how that dose can affect snail and parasite life histories. For example, ingesting many eggs may increase the likelihood of a snail becoming infected; however,

it may also decrease infected snail survival, resulting in reduced parasite transmission to the next host. In contrast, exposures of snails to few eggs may reduce the likelihood of parasites establishing in snail hosts. Here we test the hypotheses that the number of *H. eccentricus* eggs with developed miracidia exposed to *Physa acuta* snails affects (1) snail survival, (2) snail reproduction, and (3) the production of cercariae.

MATERIALS AND METHODS

Snail colonies were established in the laboratory following the protocol of Bolek and Janovy (2007). Briefly, to establish snail cultures, a few hundred physid snails were collected with a dip-net during March of 2012 from Pawnee Lake, Lancaster County, Nebraska (40º51.589'N, 96º53.468'W), placed in 18.9 L buckets with pond water and portable aerators, and brought back to the laboratory at Oklahoma State University. In the laboratory, field-collected snails were allowed to lay eggs in plastic 18.9 L buckets filled with dechlorinated water and all eggs were transferred to 37.85 L aquaria. Aquaria were filled with dechlorinated tap water and aerated with under gravel filters covered with approximately 3 cm of gravel to which a hand full of coral gravel was added as a calcium source. Each aquarium was given two 70 ml plastic containers and a cement brick (30 x 13 x 5cm) as egg laying substrates. All snail colonies were maintained under a 12:12 light-dark photoperiod at 24 ± 1 C. Hatched snails were fed a sprinkle of Tetra Min® fish flakes every week and provided fresh green leaf lettuce ad libitum. Representative hatched snails were identified as belonging to *P. acuta* based on penile morphology according to Wethington and Lydeard (2007).

Adults of *H. eccentricus* were recovered from the eustachian tubes of American bullfrogs (*Lithobates catesbeianus* = *Rana catesbeiana*) collected from Neven's Pond, Keith County, Nebraska (41º12.426'N, 101º24.510'W). Gravid adult worms were identified to species based on location in the amphibian host and the relative size of the abopercular filaments and egg length according to Bolek et al. (2010). To extract trematode eggs, 10 gravid *H. eccentricus* were shredded with forceps and all eggs were pooled in a 70 ml plastic jar containing 35 ml of dechlorinated tap water and loosely covered with a lid. Prior to use for snail exposures, all trematode eggs were kept in a cool (20 C), shaded environment for 3 mo to allow for miracidial development. Voucher specimens of field-collected adult *H. eccentricus* (accession numbers: 19167-19168) removed from the eustachian tubes of *L. catesbeianus* and of laboratory reared snails (accession numbers: 15419-15420) have been deposited in the Museum of Southwestern Biology: Division of Parasitology, University of New Mexico, Albuquerque, New Mexico.

A total of 144 second-generation laboratory reared snails were evenly divided into 12 groups of 12 individuals. All snails were measured (shell length to the nearest 0.5 mm) with handheld calipers prior to each group being placed in an individual 3.5 L glass bowl filled with 3 L of dechlorinated tap water and aerated. Snails were acclimated in glass bowls for a period of 1 wk, during which time, 5 snails died. Snails were fed green leaf lettuce ad libitum for 5 days during the acclimation period. To ensure that excessive snail feces production did not obstruct our detection of non-ingested *H. eccentricus* eggs during individual snail exposures (see below), the water was changed and all food was removed from acclimation bowls 2 days prior to snail exposure.

After the 7 day acclimation period, all snails were removed from bowls and each individual snail was placed in a 6 ml well (containing 5 ml of dechlorinated tap water), in plastic cell culture plates, to allow each individual snail to feed on a specified number of *H. eccentricus* eggs. Prior to snail exposures, all *H. eccentricus* eggs were visually inspected under a dissecting microscope and only those containing developed miracidia were pipetted into 6 ml wells. The number of *H. eccentricus* eggs given to individual snails included 0 eggs (control), 1, 3, 5, 10, 15, 20, 25, 30, 35, 40, and 50 eggs. After a 48 hr exposure, all snails were removed from individual wells and grouped back into their respective bowls. Individual wells were visually inspected for any remaining trematode eggs; all snails appeared to have ingested their entire egg dose and empty eggs were observed in snail feces. No additional snails died during this time. All exposed snails were provided fresh green leaf lettuce ad libitum and water was changed every 7 days in each bowl. The prepatent period of *H. eccentricus* in *Physa* spp. snails ranges from 28-32 days post exposure (DPE) (Bolek et al., 2010). Thus, at 28 DPE, individual snails that survived were measured as previously described and moved back into individual 6 mL wells. After 28 DPE, all surviving snails were observed daily for the presence of snail eggs and *H. eccentricus* cercariae until 40 DPE at which point all snails either died, reproduced, or shed cercariae. Generalized linear models were conducted in R (R Development Core Team, 2010) and figures were made using the function *logi.hist.plot* (de la Cruz Rot, 2005). The chi-square test for independence was calculated to compare proportions of reproductively active snails among groups of control, exposed and not infected, and infected snails (Sokal and Rohlf, 1981).

RESULTS AND DISCUSSION

Snail shell length did not significantly deviate from random between bowls prior to *H. eccentricus* egg exposure ($F_{11,130} = 0.68$, $P = 0.76$; mean ± SD: 5.16 ± 0.94; range: 3 - 7). Fifty-five exposed snails died prior to the completion of the prepatent period (28 DPE). In total, 33 of the 77 (42.8%) surviving snails shed cercariae, with the mean snail prepatent period being 28.5 ± 0.9 SD (range: 28-31). Neither snail size 28 DPE ($F_{1,31} = 0.01$, $P = 0.94$, Adj. $R^2 = -0.03$) or trematode egg exposure dose ($F_{1,31} = 0.67$, $P = 0.42$, Adj. $R^2 = -0.01$) significantly affected the prepatent period. However, we did not isolate snails until 28 DPE and any effects on potential shedding events on the front end of this range could have been missed. Only 9% (3/33: 2 exposed to 20 eggs, 1 exposed to 25 eggs) of infected snails shedding cercariae produced a single clutch of eggs between 28 and 40 DPE. In contrast, 67% (6/9) of surviving control snails produced at least one clutch of eggs over the same time period. Additionally, 83% (25/30) of surviving snails exposed to 1-5 *H. eccentricus* eggs and 22% (2/9) of surviving snails exposed to 10 *H. eccentricus* eggs, which did not produce cercariae, reproduced.

The number of trematode eggs to which snails were exposed had a significant negative effect on snail survival through the prepatent period (28 DPE), where the odds of snail survival was 0.94 per trematode egg (Wald $Z_{1,137} = -5.01$, $P < 0.001$; Fig. 3.1A). Although no snails died during the 4 days they were without food (last 2 days of acclimation period and 2 day trematode exposure), snail survival could have potentially been influenced by this 4 day event. Similarly to survival, the number of trematode eggs had a significant negative effect on snail reproduction, where the odds of snail reproduction was 0.85 per trematode egg (Wald $Z_{1,75} = -3.92$, $P < 0.001$; Fig. 3.1B). In

contrast, the number of trematodes eggs significantly increased the probability of a snail shedding cercariae, where the odds of cercariae shedding was 1.64 per trematode egg (Wald $Z_{1,75} = 3.69$, $P < 0.001$; Fig. 3.1C). Although higher exposures resulted in more infections, only 4 shedding snails survived to 40 DPE, including a single snail in the 10, 15, 20, and 25 egg exposure groups. When exposed to 30 or more eggs, all shedding snails died prior to 40 DPE. As a result, the mean number of days that snails shed cercariae was significantly lower with increasing trematode egg doses (Fig. 3.2; $F_{1,6} = 8.06$, $P = 0.03$; Adj. $R^2 = 0.50$). Additionally, there was a significant negative relationship between the number of trematode eggs exposed to snails and snail size 28 DPE ($F_{1,75} = 3.96$, $P = 0.05$); however, the relationship was weak (data not shown; $\beta = -0.22$, Adj. $R^2 = 0.04$).

The results of our study clearly demonstrate that exposure dose of *H. eccentricus* eggs affects (1) snail survival, (2) snail reproduction, and (3) cercariae production. Snails exposed to fewer *H. eccentricus* eggs were much more likely to survive and reproduce than snails exposed to higher numbers of *H. eccentricus* eggs. Interestingly, there appears to be threshold relationships for snail reproduction and cercarial production for all surviving snails 28 DPE in response to *H. eccentricus* egg dose exposure. Most surviving snails exposed to less than 10 *H. eccentricus* eggs reproduced, whereas fewer surviving snails reproduced when exposed to 10-25 *H. eccentricus* eggs, and none of the surviving snails reproduced when exposed to 30 or more *H. eccentricus* eggs. In direct contrast, surviving snails exposed to less than 10 *H. eccentricus* eggs did not produce cercariae, surviving snails exposed to 10-15 *H. eccentricus* eggs showed variation in cercarial production, and all surviving snails exposed to 20 or more *H. eccentricus* eggs produced

cercariae. However, some snails (4/18) that obtained patent infections through lower egg exposures (10-25) survived 40 DPE. In contrast, there was 100% snail mortality before 40 DPE for snails exposed to 30 or more eggs. These results suggest that higher doses of miracidia can not only induce snail mortality prior to cercarial release, but can also shorten the patent period, which could potentially reduce the probability of infecting the next host in the life cycle. Given that we ended the study at 40 DPE, the number of days snails shed cercariae was truncated for 4 individual snails (all in the lower egg dose groups) which likely dampened the observed, significant effect (Fig. 3.2).

Our observations correspond to the general patterns reviewed by Sorensen and Minchella (2001) where a higher dose of miracidia increased the incidence of snail mortality. Additionally, the latter authors indicated that, in general, a higher exposure dose of trematode miracidia increases the chances of gigantism in snail hosts. In contrast, we observed significantly smaller snail sizes at higher egg doses, suggesting that gigantism is not occurring in our system. Instead, our observations could be the result of increased mortality prior to 28 DPE of larger snails exposed to higher doses (which were not measured), or higher exposure intensities could simply result in decreased snail growth.

After 28 DPE, we observed a lack of snail reproduction in 91% of our snails with patent infections, suggesting that castration may occur in this snail-trematode system. Because we did not crush any of the infected snails for the presence or absence of gonads, we cannot confirm whether these patterns are a result of true castration. However, castration has been suggested to occur in this system (Esch et al., 1997). Importantly, 3 snails (9%) with patent infections produced eggs between 28 and 40 DPE

indicating that not all snails stop reproduction when infected with *H. eccentricus*. In contrast, 67% of control snails produced eggs and 29.2% of snails exposed to miracidia, but which did not shed cercariae, produced eggs. Reproduction was significantly different among these groups ($\chi^2 = 11.214$, df = 2, $P = 0.004$) and all pairwise comparisons between groups were significantly different ($P < 0.05$).

Interestingly, and in contrast to our results, Zelmer and Esch (2000) exposed snails (*H. anceps*) to egg-bound miracidia (*H. occidualis*) and observed a significant, positive, linear relationship between the number of *H. occidualis* eggs ingested by snails and the proportion of snails with patent infections. This suggests there are differences between the host snail species, congeneric parasite species, or their interactions. For example, the infectivity of *H. occidualis* eggs and/or the pathogenicity of *H. occidualis* rediae could be greater than that of eggs and/or rediae of *H. eccentricus* (Esch et al., 1997). Additionally, the immune systems of the 2 hosts (*H. anceps* and *P. acuta*) could differ (Esch et al., 1997). It is possible that the eggs used in our study were less infective because of their age, but infectivity tends to decrease only slowly in miracidium-bound eggs (Zakikhani and Rau, 1998) and we only used eggs that had well-developed miracidia. It is also possible that differences in snail diet and metabolism could have played a role (Sandland and Minchella, 2003). Capture-mark-recapture studies have demonstrated *Helisoma* spp. live a more sedentary lifestyle than highly vagile and metabolically demanding *Physa* spp. (Goater et al., 1989; Snyder and Esch, 1993; Esch et al., 1997; Negovetich and Esch, 2008).

Zelmer and Esch (2000) found as few as 2 and 4 *H. occidualis* eggs resulted in over 30% and 50% of the snails obtaining patent infections, respectively. Additionally,

not all the *H. anceps* snails became infected at doses up to 16 *H. occidualis* eggs whereas exposure of *P. acuta* to more than 15 *H. eccentricus* eggs resulted in 100% prevalence. Zelmer and Esch (2000) suggest the linear relationship between *H. occidualis* dose and infection prevalence in *H. anceps* was not a result of host immunity. In contrast, we found a threshold (10 *H. eccentricus* eggs: 55.5% prevalence; 15 eggs: 71.4% prevalence) at which the infection prevalence rapidly transitions from 0% to 100%. Although these patterns suggest the immune system of *P. acuta* may play a role, we did not collect immunological data, and thus, have no direct evidence to support this hypothesis.

Zelmer and Esch (2000) also found a weak ($R^2 = 0.17$), significant, positive relationship between snail length and prepatent period which we did not observe. The variation of prepatent period in *P. acuta* snails was extremely low and thus we were unable to adequately test for this trend. Additionally, the significance of this relationship in *H. anceps* appears to be driven by the extreme values of the single smallest snail and the 2 largest snails (Zelmer and Esch, 2000; Fig. 3.2) and thus, should be tested further. These contrasting results suggest that transmission patterns can differ between closely related parasite species.

In conclusion, our laboratory infections suggest there is a distinct tradeoff where higher doses of trematode eggs may be more likely to result in a patent infection, but also increases the odds of killing the host before releasing cercariae, or, early on in the infection. On the other hand, low infection intensities may not reduce snail host fitness, but may not be enough to establish an infection. Although we report fewer shedding days for snails exposed to higher trematode egg doses, there may be potential differences in

the number of cercariae released per day, which we did not record. For example, although a heavily infected snail might die within a day or so, it could shed more cercariae than a lightly-infected snail that only releases a few cercariae over a longer period of time. This would be an interesting avenue for further study. Overall, this study gives insight into the potential *H. eccentricus* egg doses snails experience in nature and has important implications for future laboratory studies using domesticated trematode life cycles.

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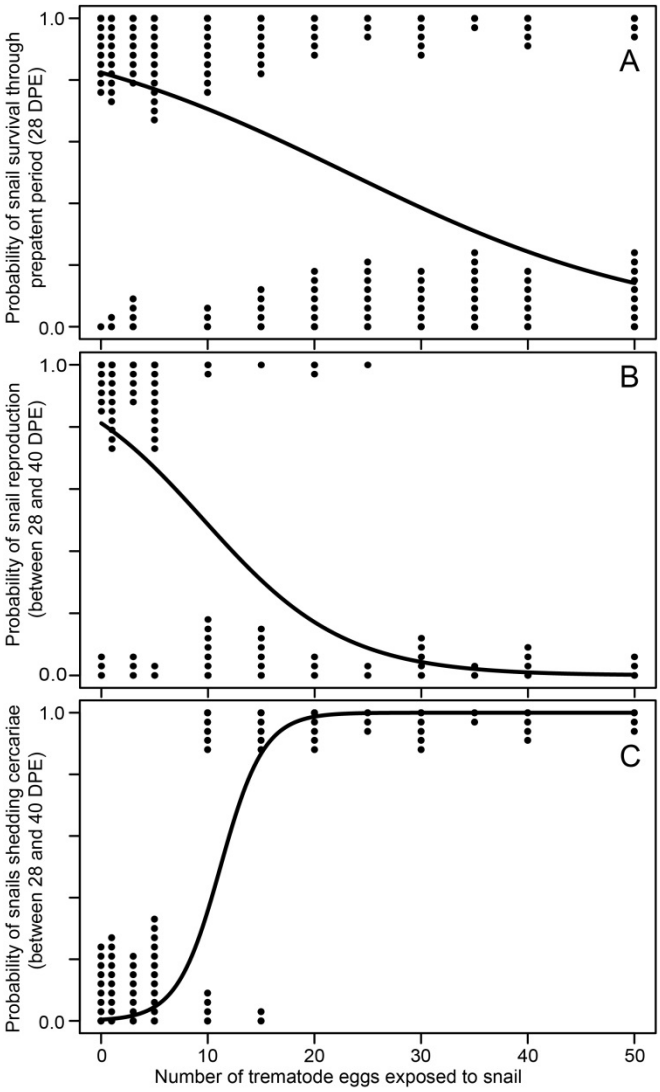

Figure 3.1. Probability (plotted lines) of (A) snail survival through the prepatent period (28 days post exposure [DPE]), (B) snail reproduction from 28-40 DPE, and (C) cercariae shedding (infection prevalence was overlaid when variation was present) in relation to exposure dose of *Halipegus eccentricus* eggs to *Physa acuta* snails. Only snails that survived 28 DPE were used to determine snail probability of reproduction and cercariae shedding. Circles on the top axes represent individual snails in each trematode egg exposure treatment group that survived the prepatent period, reproduced between 28 and 40 DPE, or shed cercariae; respectively. Circles on the bottom axes represent individual snails that did not survive the prepatent period, did not reproduce between 28 and 40 DPE, or did not shed cercariae, respectively.

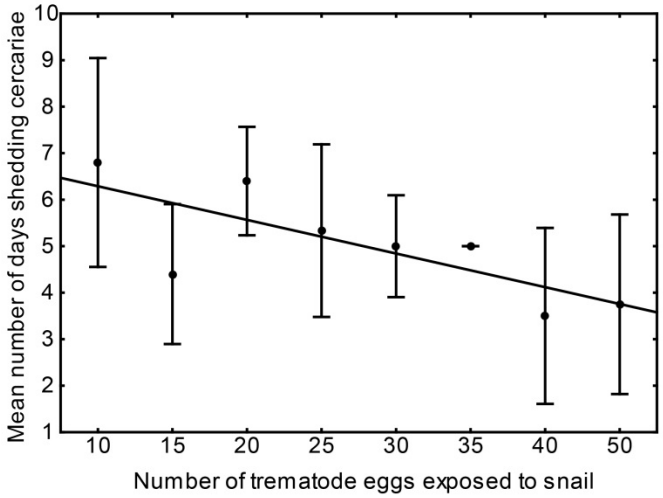

Figure 3.2. The mean (± SE) number of days that snails shed cercariae in relation to the number of trematode eggs to which they were exposed. The line represents the linear regression fit of the means. Only the snails that released cercariae were used to calculate means (see top row of Fig. 3.1C for sample sizes in each group).

CHAPTER IV

ATRAZINE REDUCES THE TRANSMISSION OF AN AMPHIBIAN TREMATODE BY ALTERING SNAIL AND OSTRACOD HOST-PARASITE INTERACTIONS

Gustafson, KD, JB Belden, and MG Bolek (2016) Atrazine reduces the transmission of an amphibian trematode by altering snail and ostracod host-parasite interactions. Parasitology Research 115:1583–1594. doi:10.1007/s00436-015-4893-1

This research was original and was conducted under the advisement of my advisor, Matthew Bolek, and my committee member, Jason Belden. This chapter has been published in *Parasitology Research*.

ABSTRACT

Trematodes are ubiquitous members of aquatic environments, have many functional roles in ecosystems, and can cause diseases in humans, livestock, and wild animals. Despite their importance and reports of parasite population declines, few studies have concurrently assessed the effects of aquatic contaminants on multiple hosts, multiple parasite life cycle stages, and on transmission-related host-parasite interactions. Here, we test the effects of environmentally-relevant concentrations of the herbicide atrazine (0, 3, 30 μg/L) on the establishment and development of an amphibian trematode (*Halipegus eccentricus*) in a first-intermediate snail host (*Physa acuta*) and in a second-intermediate ostracod host (*Cypridopsis* sp.). Additionally, we test the interactive effects of atrazine and parasitism on snail and ostracod survival. Our results indicate atrazine negatively affects trematode transmission by altering snail and ostracod host-parasite interactions. Although atrazine did not affect the survival of uninfected snails alone, atrazine acted synergistically with parasitism to reduce the longevity of infected snails. As a result, the number of cercariae (i.e., larval trematodes) produced by snails was 50.7% (3 μg/L) and 14.9% (30 μg/L) relative to controls. Atrazine exhibited direct negative effects on ostracod survival at 30 μg/L. However, when ostracods were also exposed to trematodes, the negative effects of atrazine on survival were diminished. Although infected ostracod survival remained high, trematode development was significantly reduced, resulting in reduced infectivity of metacercariae (i.e., non-gravid adult cysts infective to definite host) to 32.2% (3 μg/L) and 28.6% (30 μg/L) relative to the controls. The combination of reduced cercaria production and reduced metacercarial infectivity in the 3 and 30 μg/L atrazine treatment groups reduced the net number of infective worms produced to 16.4%

and 4.3% (respectively) relative to the control. These results demonstrate the complex nature of pesticide effects on trematode infections and indicate that trematodes can affect their first- and second-intermediate hosts differently under different pesticide concentrations. Our work has broad implications for parasite transmission and conservation, and provides a testable mechanism for understanding trematode population declines in contaminated wetlands.

INTRODUCTION

Historically, much effort has been spent attempting to disrupt parasite life cycles in order to reduce disease incidence in hosts (Bowman 2006). However, it is becoming increasingly evident that obligate symbiotic organisms, including parasites, are declining at a much faster rate than their free-living counterparts (Dunn et al. 2009). This is of particular concern given that parasitism is the most common lifestyle on earth and makes up an estimated half of the planet's biodiversity (Dobson et al. 2008; Poulin and Morand 2000; Price 1980). Additionally, the contribution of parasites to the free-standing biomass, coupled with their effects on hosts, makes parasites major contributors to the functions and dynamics of ecosystems (Hatcher et al. 2012; Kuris et al. 2008; Preston et al. 2013). Despite concerns over the loss of parasite biodiversity (e.g., Dunn et al. 2009; Russell et al. 2015), parasites have been largely ignored by conservation biologists and the scientific community, in general (Gómez and Nichols 2013; Holmes 1993; Rózsa 1992; Thompson et al. 2010).

It is now well-established that anthropogenic disturbances affect parasite life cycles (Patz et al. 2000), particularly those of digenetic trematodes (Blanar et al. 2009;

Lafferty 1997; McKenzie 2007; Rohr et al. 2008a; Rohr et al. 2008b). Digenean trematodes typically exhibit complex 3-host life cycles including an obligate first-intermediate molluscan host, second-intermediate vertebrate or invertebrate host, and a vertebrate definitive host (Shoop 1988). Molluscs get infected by ingesting or being penetrated by miracidia which develop and asexually produce cercariae (Shoop 1988). Cercariae infect second-intermediate hosts, encyst to become metacercariae, and metacercariae must be ingested by the definitive host to mature and produce eggs (Shoop 1988). Land use practices can cause definitive hosts to shift habitats which then affects the source and spatial deposition of trematode eggs (Esch et al. 2001; Fredensborg et al. 2005; Hechinger and Lafferty 2005; Koprivnikar and Redfern 2012). Additionally, during the aquatic portion of the life cycle, aquatic hosts and aquatic trematode stages can be exposed to pesticides that may affect hosts, parasites, or their interactions (Koprivnikar et al. 2007a; Koprivnikar et al. 2007b; Pietrock and Marcogliese 2003; Schotthoefer et al. 2011). Several recent studies assessing the effects of chemicals on trematode life cycles have focused on the transmission of amphibian trematodes because of concerns of the potential role parasites may play in amphibian population declines (Koprivnikar et al. 2012; Szuroczki and Richardson 2009). Field studies have observed lower trematode species richness in amphibians inhabiting agricultural areas compared to those inhabiting less-impacted areas (King et al. 2008; King et al. 2010; King et al. 2007; Koprivnikar et al. 2006a; Marcogliese et al. 2009; Schotthoefer et al. 2011). Most authors attribute the loss of trematode species diversity in amphibians from contaminated sites a result of definitive hosts avoiding contaminated sites and not depositing trematode eggs and miracidia. However, this could be compounded by reduced transmission within aquatic

environments for chemically-sensitive trematode species or from a reduced abundance of chemically-sensitive intermediate host species.

Interestingly, for the amphibian trematode species that remain in contaminated sites, several authors report increased parasite intensities within amphibian hosts (King et al. 2010; King et al. 2007; Marcogliese et al. 2009; Rohr et al. 2008b; Schotthoefer et al. 2011), which has been suggested to be a result of eutrophication and host immunosuppression. Eutrophication can increase the local abundance of snails and the production of cercariae via increased nutrient availability (Bernot 2013; Johnson et al. 2007), whereas pesticide-induced immunosuppression can increase the susceptibility of potential hosts to infections (Christin et al. 2003; Kiesecker 2002; Koprivnikar et al. 2007b; Rohr et al. 2008a). Interestingly, Koprivnikar et al. (2007a) observed a lower proportion of snails transmitting trematode cercariae in agricultural ponds compared to nonagricultural ponds which is contrary to the higher prevalence and intensities previously observed in frog hosts at these locations (Koprivnikar et al. 2006a). Although their observations could be a result of definitive host avoidance (i.e., low source of trematode eggs), it could also be a result of pesticide effects on miracidial infectivity to snails, induced snail host mortality through the interactive effects of pesticides and parasitism, or a combination of these factors (Marcogliese and Pietrock 2011; Pietrock and Marcogliese 2003). Hypotheses pertaining to the direct and indirect effects of pesticides on trematode transmission need to be explored further.

Thus far, few studies have addressed pesticide effects on trematode life cycles. This is not surprising because, besides the medically-important *Schistosoma* spp. and various echinostome flukes, there are few trematode life cycles that have been completed

in the laboratory (Loker 2010). Thus far, laboratory experiments attempting to isolate pesticide effects on trematode transmission have focused on the hatching success of miracidia (e.g., Raffel et al. 2009), the production of cercariae (e.g., Hock and Poulin 2012), cercarial longevity (e.g., Koprivnikar et al. 2006b), and host immunosuppression (e.g., Christin et al. 2003). Additionally, studies on cercariae released from snails typically use infected field-collected snails with an unknown pesticide exposure history. Although these studies, along with others, have all identified specific effects on hosts or specific effects on parasites, simultaneous exposures to hosts and parasites, which is the environmentally-relevant condition, are lacking. Given that most trematode life cycles are aquatic, especially the stages from egg to metacercaria (Shoop 1988), it is essential to assess the impacts of chemicals on multiple life cycle stages, on multiple aquatic hosts, and on their host-parasite interactions.

Here, we assess the impacts of the herbicide atrazine on the transmission of an amphibian trematode (*Halipegus eccentricus*) from egg to metacercaria, simultaneously exposing intermediate hosts and trematodes to environmentally-relevant atrazine concentrations throughout the life cycle. We chose to study atrazine because it is one of the most commonly used and detected agricultural pesticides in North America (Gilliom 2007; Graymore et al. 2001; Solomon et al. 1996) and it has been implicated to increase transmission of some amphibian trematodes to their amphibian hosts (Rohr et al. 2008b). Additionally, culturing techniques to complete the life cycle of *H. eccentricus* in the laboratory have been developed, providing an exceptional model for studying the impacts of extrinsic factors on trematode transmission (Bolek et al. 2010). *Halipegus eccentricus* exhibits a 3- to 4-host life cycle including true frog (i.e., Ranidae) definitive hosts,

obligate snail (*Physa* spp.) first-intermediate hosts, and a variety of obligate microcrustacean species as second-intermediate hosts (Bolek et al. 2010). Additionally, several odonate species can serve as paratenic hosts (Bolek et al. 2010; Wetzel and Esch 1996). We obtained adult worms of *H. eccentricus* from the eustachian tubes of bullfrogs (*Lithobates catesbianus*), infected laboratory-raised first-intermediate snail hosts (*Physa acuta*) with egg-bound miracidia, and then infected laboratory-raised second-intermediate ostracod hosts (*Cypridopsis* sp.) with cercariae in 3 environmentally-relevant atrazine concentrations (0, 3, 30 µg/L). Specifically, we test the effects of atrazine on infection establishment in snails (*P. acuta*), production of cercariae, infection establishment in ostracods, and the infectivity of metacercariae to amphibian hosts. Additionally, we test the interactive effects of atrazine and parasitism on the survival of snail and ostracod hosts. Results of this study provide insights into how pesticides affect trematode transmission, host-parasite relationships, and address a potential mechanism for the observed loss of trematode species diversity in contaminated aquatic ecosystems.

METHODS

Chemicals

Atrazine (2-chloro-4-ethylamino-6-isopropylamino-s-triazine, 98% purity) was obtained from Chem Service, Inc. (West Chester, PA). The solvent, acetone, was obtained from Fisher Scientific (Waltham, MA) and was pesticide grade or better. Dechlorinated water was obtained by carbon filtration of Oklahoma State University tap water (pH: 7.4 – 7.9, Hardness: 80 – 100 mg calcium carbonate/L) and was used in all exposure tests and for maintenance of experimental cultures. Using gas-chromatography

mass-spectrophotometry and select ion monitoring (see quality control section for details), the dechlorinated water did not have detectable background levels of atrazine (limit of detection: <0.05 µg/L).

Snails (Physa acuta)

Snail colonies were established in the laboratory following the protocol of Gustafson and Bolek (2015). Briefly, a few hundred snails of the family Physidae were collected in March of 2013 from Pawnee Lake, Lancaster County, Nebraska (40º51.589'N, 96º53.468'W). Field collected snails were allowed to lay eggs in plastic 19 L (5 gallon) buckets filled with dechlorinated water and all eggs were transferred to 38 L (10 gallon) aquaria. Aquaria were filled with dechlorinated tap water and aerated with under gravel filters covered with approximately 3 cm of gravel to which a hand full of coral gravel was added as a calcium source. Each aquarium contained two 70 ml plastic containers and one brick as egg laying substrates. All snail colonies were maintained under a 12:12 light-dark photoperiod at room temperature. Hatched snails were fed a sprinkle of Tetra Min® fish flakes every week and a combination of fresh and frozen green leaf lettuce *ad libitum*. Representative hatched snails were identified as belonging to *Physa acuta* based on penial morphology according to Wethington and Lydeard (2007) and Gustafson et al. (2014). A total of 720 second generation laboratory reared snails were measured (shell length in mm to the nearest 0.25 mm) with a handheld calipers prior to parasite exposure.

Trematodes (Halipegus eccentricus)

Three adults of *Halipegus eccentricus* were recovered from the eustachian tubes of American bullfrogs (*L. catesbeianus*) collected from Neven's Pond, Keith County,

Nebraska (41º12.426'N, 101º24.510'W) following the collection and identification procedures of Gustafson and Bolek (2015). After the removal of worms, frogs were released at the site of capture. In brief, the 3 gravid *H. eccentricus* were shredded with forceps and eggs were pooled and kept in a loosely-covered 70 ml plastic container with dechlorinated water in a cool (20º C), shaded environment for 3 months to allow for miracidial development.

Snail-trematode egg exposures to atrazine

Because snails and trematode eggs would concurrently experience similar aqueous chemical concentrations in nature, our approach was to expose snails and trematode eggs separately to 0, 3, or 30 µg/L atrazine prior to exposing snails to trematode eggs. Chronic exposures below 100 µg/L atrazine are considered ecologically-relevant (Graymore 2001). We chose to use 0, 3, and 30 µg/L because they are ecologically-relevant and do not directly affect snail fitness (Gustafson et al. 2015). Additionally, to allow comparison to the singular effects of atrazine to snails, snails were also exposed to atrazine in the absence of trematode eggs. The experiment was conducted as a complete two-factor design with presence or absence of trematode (+/-) and 3 atrazine treatments of trematode and snails (0, 3, or 30 µg/L), resulting in 6 treatments with 120 snails per treatment (720 snails).

Prior to use in snail exposures, eggs of *H. eccentricus* were visually inspected under a dissecting microscope and only eggs containing miracidia were used for snail exposures. Selected eggs (*H. eccentricus*) were placed 10 per well in 360 wells of multi-well polystyrene plates (15ml/well, 6 wells/plate) with 10 ml of dechlorinated tap water (solvent control; 0.00003% acetone), 3, or 30 µg/L atrazine (120 wells/concentration with

trematodes; 120 wells/concentration without trematodes). All wells on the same plate received the same treatment and treatment of eggs was a static exposure of 7 d. Well plate covers were placed on the wells to minimize evaporation. Gustafson and Bolek (2015) demonstrated variation in establishment of an infection (between 30-80% prevalence) when snails were exposed to 10-15 eggs of *H. eccentricus*; egg exposures below 10 did not result in infections whereas egg exposures above 15 resulted in 100% prevalence. Thus, if atrazine affects infection dynamics from egg to snail, we would expect to detect it between the 10 and 15 egg dose. We chose to expose half of the snails (N = 120 snails/concentration) to 10 trematode eggs each; the other 360 snails were not exposed to trematode eggs (but were still exposed to 1 of 3 atrazine concentrations).

Simultaneously to treatment of eggs, snails (720 individuals) were exposed to atrazine in thirty 3.5 L bowls filled with 3 L of dechlorinated tap water that were randomly spiked with 1 of 3 nominal concentrations of atrazine (0, 3, and 30µg/L). Control (0µg/L atrazine) bowls were only treated with an equivalent acetone volume (100µL; 33µg/L or 0.00003%). Bowls serve as convenient and temporary housing for snails during trematode development and not as experimental units. Snails were evenly (N=24/bowl) grouped in bowls and fed green leaf lettuce *ad libitum* for 5 days. After this 5 day period, the water with the appropriate atrazine concentration was renewed and all food was removed for the last two days of pre-exposure to clear snail gastrointestinal tracts allowing better visualization to determine if snails consumed the 10-egg trematode dose; this method has been previously shown to not affect snail survival (Gustafson and Bolek 2015).

The solvent, acetone, is not toxic to snails at low levels (0.0002 %; Rohr et al. 2008a) and only mildly reduces snail fecundity at concentrations of 0.1 % (Bluzat et al. 1979; Hutchinson et al. 2006). Additionally, the United States Environmental Protection Agency, among others, indicate that acetone is a recommended solvent at concentrations below 500 µg/L (for acute toxicity tests) and 100 µg/L (for chronic toxicity tests) because it has a high evaporation rate and is 100% volatile (i.e., can quickly and completely evaporate from the water) (EPA 1975; LeBlanc and Surprenant 1983). As a result, our experiments did not include negative controls (i.e., water only, no acetone), given the extremely low amount of acetone used, and its presence in water bowls for minutes prior to complete evaporation, we did not expect acetone to affect our results and conclusions.

After snails and trematode eggs were individually exposed, snails were removed from bowls and individually placed in one of the 15 ml wells containing 10 ml of dechlorinated tap water with respective atrazine concentrations; half of the wells at each concentration contained 10 eggs of *H. eccentricus* from the previously described exposure and the other half contained only atrazine. Well plate covers prevented snails from escaping. After 48 hours, all snails were removed from individual wells and, within parasite exposure groups, randomly placed back into bowls based on treatment level (N = 24 snails/bowl). Individual wells were visually inspected for any remaining trematode eggs and all snails appeared to have ingested their entire dose of eggs. All snails were provided fresh and frozen green leaf lettuce *ad libitum*; water and relevant atrazine concentrations were replenished twice weekly (Monday and Friday) in each bowl. Any egg masses laid were removed during water changes. This second tank period maintained

atrazine exposure to snails and trematodes, but was ended prior to shedding of cercariae and thus does not interfere with endpoint measurement of individual snails.

The prepatent period of *H. eccentricus* in snails from the genus *Physa* ranges from 28-32 days post parasite exposure (DPPE) (Bolek et al. 2010; Gustafson and Bolek 2015). Thus, at 25 DPPE the length of each snail was measured as previously described and snails were transferred back into individual 15 mL wells containing 10 mL of renewed treatment water. Well plate covers were placed on each 6 well plate to prevent snails from escaping. All snails that shed cercariae began releasing cercariae within 31 DPPE (N= 47; mean DPPE ± SD = 28.3 ± 0.73; range = 28-31). When cercariae were detected, the snail was moved to a new well containing 10 ml of dechlorinated tap water with respective atrazine concentrations. Similarly, a reference snail of similar size (± 0.25 mm), never exposed to trematode eggs, and from the same atrazine concentration, was moved into its own well. Each infected and reference snail was provided a small piece of frozen green leaf lettuce and was transferred to a new well (with renewed water and atrazine concentrations) every 24 hours for the duration of its life. Prevalence was recorded as the percent of snails with patent infections (i.e., releasing cercariae).

Each infected snail and reference snail was treated as an experimental unit. For each infected snail, we recorded the number of cercariae shed every 24 hours. Snails not exposed to trematodes served as a comparison for survival analyses. We used an analysis of variance (ANOVA) to test the effects of atrazine and parasitism on the number of days snails survived. After an individual snail died, it was dissected to verify trematode infection status. We used repeated measures ANOVAs to test the interactive effects of atrazine and time on the production of cercariae. We also used ANOVAs to test the

effects of atrazine on the number of snails that became infected (i.e., prevalence), the number of days snails shed cercariae, and the overall number of cercariae produced per snail. If significant, a Tukey's Unequal N HSD post hoc test was used to determine significant differences between groups. The total number of cercariae produced per snail did not meet the assumptions of an ANOVA and thus we used a nonparametric Kruskal-Wallis median test and multiple comparisons of mean ranks post hoc tests (z-tests). Statistics were conducted using STATISTICA v10 (StatSoft, Inc. 2011). Figures were created using Adobe Illustrator CS6 (Adobe Systems, Inc. 2012).

Ostracod-cercariae exposures to atrazine

Because ostracods and cercariae both could experience similar concurrent atrazine exposures in nature, our approach was to expose ostracods and cercariae separately in 0, 3, or 30 µg/L atrazine prior to exposing ostracods to cercariae. We tested and controlled for any potential effects of trematode atrazine exposure history on ostracods or trematodes by exposing ostracods to cercariae sourced from snails in control atrazine concentrations and by exposing control ostracods (not treated with atrazine) to cercariae sourced from snails in higher relative atrazine concentrations as well. Overall, this resulted in 4 treatment groups with multiple atrazine concentrations tested in a nested design including: 1) ostracods not exposed to cercariae (N = 48 ostracods per treatment group; treatment groups include ostracods in 0, 3, and 30 µg/L atrazine), 2) ostracods exposed to cercariae sourced from snails from the same concentration (N = 48 ostracods per treatment group; treatment groups include ostracods and cercariae in 0, 3, and 30 µg/L), 3) ostracods exposed to cercariae sourced from control (0 µg/L atrazine) snails (N = 48 ostracods per treatment group; treatment groups include ostracods from 0, 3, and 30

μg/L atrazine), and 4) ostracods in control concentration exposed to cercariae sourced from snails from higher atrazine concentrations (N = 48 ostracods per treatment group; treatment groups include cercariae from 3 and 30 μg/L atrazine). Overall, this experimental design resulted in a sample size of 480 ostracods.

To control for age of cercariae, all the cercariae were pooled from snails within each atrazine treatment group (0, 3, or 30μg/L) the first day of shedding (28 DPPE) and kept at room temperature in their respective atrazine concentrations. The 480 ostracods (*Cypridopsis* sp.) were collected the same day from laboratory cultures maintained in 38 L aquaria (not treated with atrazine) and randomly maintained in 1 of 3 atrazine concentrations. Seven days later, ostracods in each treatment group were individually exposed to three 7-day-old cercariae. Although infectivity generally declines with age (Wetzel and Esch 1995), cercariae of *Halipegus* have been reported to be infective for up to 60 days (Shostack and Esch 1990). Forty-eight ostracods in each atrazine concentration were not exposed to cercariae. Wells were the same size as those used to expose snails (15 mL) but contained 14 mL of treatment water.

After 3 days, the cercariae had all appeared to have been eaten by the ostracods and a small piece of frozen green leaf lettuce was added as ostracod food to each well. Lettuce was replenished as needed. Atrazine exposures were static and the water was not renewed during this experiment. Twenty-two DPPE, individual ostracods were gently crushed between a slide and cover slip to determine infection status (Appendix 4.1A). The number of worms within each ostracod and the presence or absence of a skirt-like bladder on each worm was recorded. Experiments indicate worms that retain the skirt-like bladder are unable to infect the odonate paratenic host or the frog definitive host

(Appendix 4.2A); whereas worms that pinch off the skirt-like bladder after 15-19 days of development in *Cypridopsis* sp. are infective to the next host in the life cycle (Bolek et al. 2010; Zelmer and Esch 1998a). Thus, the percent of worms that pinched off the skirt-like bladder in each treatment serves as a proxy for infectivity. To quantitate the overall effect of atrazine on the transmission of *H. eccentricus*, we calculated the net number of worms that were produced and became developmentally infective by counting the number of cercariae produced from snails and multiplying that number by the proportion of metacercariae that pinched off the skirt-like bladder.

Because of limited supply of cercariae, we were unable to expose ostracods in each atrazine treatment to cercariae from each atrazine treatment, resulting in an incomplete factorial design. Thus, we used nested ANOVAs (cercaria source nested in atrazine exposure concentration exposed to ostracod) to test the effects of cercaria exposure history on ostracods and metacercariae within ostracod treatment groups. We tested the effects of atrazine and parasite exposure on ostracod survival, the percent of ostracods that became infected (i.e., prevalence), and on the percent of worms that pinched off their skirt-like bladder (i.e., infectivity). If a significant effect was observed, a Tukey's Unequal N HSD post hoc test was used to determine significant differences between groups. Statistics were conducted using STATISTICA v10 (StatSoft, Inc. 2011). Figures were created using Adobe Illustrator CS6 (Adobe Systems, Inc. 2012).

Quality control

Water quality measurements (i.e., ammonia, DO, water temperature) were taken in bowls containing snails. Twice after the longest period between water changes (i.e., 4 d), water temperature and dissolved oxygen were randomly subsampled from 4 bowls at

each concentration using a YSI DO Professional Series Probe (Yellow Springs, OH). In the same manner, ammonia (NH3-N mg/L) was randomly subsampled once from 3 bowls at each concentration using HACH Test 'N Tube kits (Nessler method 8038) and analyzed on a HACH UV spectrophotometer (DR 5000; Loveland, CO). To verify nominal atrazine concentrations, 4 bowls at each concentration were randomly sampled once after the longest period between water changes (i.e., 4 d) and once immediately after a water change. To quantify atrazine in water, an internal standard of 100ng of atrazine-D5 (Sigma Aldrich, St. Louis, MO) was added to each sample. Atrazine was extracted from water using C18 6mL solid phase extraction cartridges (SampliQ, Agilent Technologies, Santa Clara, CA) that were previously conditioned with 5mL methanol. Atrazine was then eluted with 8mL ethyl acetate, and evaporated to 1mL using nitrogen gas. Samples were analyzed on a gas chromatograph-mass spectrometer (Agilent 5975c; Santa Clara, CA) using internal calibration and select ion monitoring (atrazine:200; atrazine- D5:205). Water quality measurements were not taken during the ostracod exposures; however, atrazine concentrations were produced following the protocols of the snail exposures and initial concentrations are not expected to be different.

RESULTS

Quality control

Atrazine concentrations were within 5.67 ± 2.39% (mean ± SD) of nominal concentrations. Refer to the Appendix 4.3A (supplementary table) for measured concentrations at each nominal concentration. In control treatments (i.e., solvent only), atrazine concentrations were below detection limit (<0.05 µg/L). Water temperature

(mean ± SD = 25.18 ± 0.36; min-max = 22.5-26.1 C), dissolved oxygen (7.07 ± 0.31; 7.05-7.19 mg/L), and ammonia concentrations (0.17 ± 0.08; 0.03-0.31 mg/L NH_3-N) remained consistent between atrazine concentrations. Refer to the Appendix 4.3A (supplementary table) for water quality data at each nominal concentration.

Snail-atrazine-trematodes

Prepatent period (i.e., 28 DPPE) survival was high (83%) and neither atrazine ($F_{2, 714} = 0.09$, $P = 0.91$), trematode exposure ($F_{1, 714} = 1.19$, $P = 0.16$), nor the interaction ($F_{2, 714} = 1.14$, $P = 0.25$) had a significant effect on the survival of snails over this 28 day duration. Although there was a general trend of fewer snails becoming infected and releasing cercariae in higher atrazine concentrations (Table 4.1), atrazine did not have a significant effect on the prevalence of *H. eccentricus* in snails ($F_{2, 310} = 1.73$, $P = 0.17$). When comparing the size of infected snails with uninfected snails of similar size, ANCOVA results indicate that snail size did not significantly affect the number of days snails survived ($F_{1, 77} = 0.0008$, $P = 0.98$) and thus size was removed from the analysis. ANOVA results of snail survival after the prepatent period indicated that atrazine did not directly affect snail longevity ($F_{2, 83} = 0.47$, $P = 0.63$). However, infection status ($F_{1, 83} = 148.38$, $P < 0.001$) and the interaction between infection status and atrazine ($F_{2, 83} = 5.62$, $P = 0.005$) significantly reduced snail longevity (Table 4.1; Fig. 4.1). Post hoc tests showed that uninfected snails lived significantly longer (64.23 ± 12.6 [SD] days) than infected snails (39.48 ± 7.13 days; Δ27.25 days). Additionally, infected snail survival was significantly lower at higher atrazine concentrations where snails died 5.8 ± 2.09 days after the prepatent period (30 µg/L) compared to 14.74 ± 6.68 days for infected control snails and 11.07 ± 7.71 days for infected snails exposed to 3 µg/L atrazine.

Time had a significant negative relationship with the number of cercariae produced per snail ($F_{22, 66} = 4.15$, $P < 0.001$). Given the short duration of snail survival past the prepatent period in the highest atrazine treatment group, we could only test the effect of atrazine on the number of cercariae produced per snail per day over 7 days. Time continued to have a significant negative effect ($F_{6, 132} = 8.45$, $P < 0.001$) on the number of cercariae produced when atrazine and the interaction of atrazine and time were included in the analysis; however, atrazine did not exhibit significant direct effects on the number of cercariae produced per snail per day ($F_{2, 22} = 2.32$, $P = 0.12$) or interactively with time ($F_{12, 132} = 1.39$, $P = 0.17$).

The number of days snails released cercariae (mean = 9.81 ± 6.05; range = 1-25) was not significantly affected by the covariate snail size ($F_{1,38} = 0.36$, $P = 0.55$), which was removed from the analysis. However, the number of days snails released cercariae was significantly affected by atrazine ($F_{2, 39} = 8.65$, $P < 0.001$, Fig. 4.2a). Although each snail did not shed cercariae each day after shedding began, the number of days each snail survived post prepatent period was significantly and positively correlated with the number of days snails shed cercariae ($R^2 = 0.77$, $P < 0.001$). Thus, as a result of living longer, infected control snails shed cercariae for significantly more days than snails from the 3 µg/L and 30 µg/L atrazine treatment groups (Table 4.1; Fig. 4.2a). The significant effect of atrazine on snail survival and the number of days snails shed cercariae significantly reduced the total number of cercariae shed throughout each snail's lifetime (Kruskal-Wallis $H = 11.84$, $P = 0.003$). As a result, snails exposed to a 3 µg/L atrazine concentration released 49.3% fewer cercariae and snails exposed to a 30 µg/L atrazine concentration released 85.1% fewer cercariae relative to the control snails (Table1; Fig.

4.2b). Pairwise comparisons of rank means indicated that control snails released significantly more cercariae per snail than snails from the 30 µg/L atrazine treatment group ($Z = 3.34$, $P = 0.002$). All other pairwise comparisons were not significant ($P > 0.05$).

Ostracod-atrazine-trematodes

Sixty-seven of the 281 (23.8%) ostracods that survived to 22 DPPE became infected with metacercariae. Three ostracods had double infections whereas all the rest had single-worm infections. Although the control ostracods had a slightly higher prevalence than ostracods in 3 and 30 µg/L atrazine, neither source of cercariae ($F_{4,274} = 1.39$, $P = 0.23$) nor atrazine ($F_{2,274} = 1.87$, $P = 0.16$) had a significant effect on prevalence of *H. eccentricus* in ostracods (Table 4.1).

Atrazine did not significantly affect the survival of cercaria-exposed ostracods ($F_{2, 329} = 0.17$, $P = 0.85$). Similarly, for ostracods exposed to cercariae, the source of cercariae (i.e., snail treatment group) did not significantly affect ostracod survival within ostracod atrazine treatment groups ($F_{4, 329} = 2.01$, $P = 0.09$). Thus, data were pooled for testing the effects of atrazine and parasite exposure on ostracod survival. Atrazine ($F_{2, 474} = 21.56$, $P < 0.001$), parasite exposure ($F_{1,474} = 4.84$, $P = 0.028$), and the interaction ($F_{2,474} = 20.41$, $P < 0.001$) significantly affected ostracod survival. Parasite-exposed ostracods had a relatively high survival (pooled mean = 83.6%) with no significant differences between atrazine concentrations (Table 4.1; Fig. 4.3). Survival was slightly higher for non-parasite-exposed ostracods in control and 3 µg/L atrazine treatment groups, however, no significant differences existed for control and *H. eccentricus*-exposed ostracods at the 0 and 3 µg/L atrazine concentrations (Table 4.1; Fig. 4.3). Post

hoc tests revealed significantly lower survival of ostracods not exposed to *H. eccentricus* in the highest atrazine concentration compared to all other treatment groups (Table 4.1; Fig. 4.3).

Within atrazine treatment groups, the developmental infectivity of metacercariae was not significantly different based on the source of cercariae ($F_{4,63} = 0.40$, $P = 0.81$). Although there were no significant differences in the number of cercariae that established infections in ostracods, atrazine had a significant negative effect on the developmental time of metacercariae and on the ability to pinch off their excretory bladder (i.e., infectivity) ($F_{2,67} = 7.09$, $P = 0.002$; Table1; Fig. 4.4). Significance of the main effects was not different when excluding worms that came from double infections.

Taking into account the total number of cercariae produced from snails in each treatment group and the significantly reduced infectivity of metacercariae in the 3 μg/L and 30 μg/L atrazine treatment groups, atrazine reduced the net number of infective worms produced through the entire study to 16.4% and 4.3% (respectively) relative to the control (Table 4.1).

DISCUSSION

The results of this study demonstrate that environmentally-relevant atrazine concentrations have the capacity to reduce transmission rates of *H. eccentricus*. Specifically, our results indicate that atrazine acts interactively with parasitism to reduce snail host survival and subsequently shortens the number of days snails release cercariae. As a result, snails produced fewer cercariae throughout their life which dramatically reduced the production of cercariae with increasing atrazine concentrations. Additionally,

atrazine exhibited negative effects on the development of metacercariae within ostracod hosts, resulting in reduced or delayed infectivity to the next host in the life cycle. Although transmission of *H. eccentricus* was not completely inhibited at higher atrazine concentrations, the combination of low cercaria production and the reduced infectivity of the remaining worms demonstrate that pesticides can negatively affect multiple aspects of a trematode life cycle.

To our knowledge, this is the first study to test the effects of atrazine on laboratory infections in snails. Although Raffel et al. (2009) tested the effects of atrazine on miracidial development and hatching, and several studies tested the direct effects of atrazine on snails (see Gustafson et al. 2015 for review of atrazine effects on snails), none of these studies tested the effects of atrazine on miracidial infectivity or prevalence in snails. The only empirical field study attempting to relate pesticide concentrations to trematode prevalence in snails was conducted by Koprivnikar et al. (2007a). Although they were unable to quantify atrazine concentrations in most ponds, they observed a higher proportion of snails shedding cercariae from reference ponds compared to agricultural ponds and suggested intra-wetland factors played a significant role in their observations. In general, and although not significant, we observed decreasing prevalence of *H. eccentricus* with increasing atrazine concentrations. Although our observed laboratory prevalence for each treatment group was lower than expected based on the results of Gustafson and Bolek (2015) who demonstrated a dose of 10 eggs of *H. eccentricus* resulted in an infection probability of >30%, prevalence in each treatment group was similar to reports for *Halipegus* species observed in nature which range from 1% to 50% for *H. eccentricus* and 3% to 57% for *H. occidualis* (Bolek et al. 2010; Goater

et al. 1989; Rhoden and Bolek 2012; Sapp and Esch 1994; Snyder and Esch 1993; Williams and Esch 1991).

Environmentally-relevant concentrations of atrazine also have little effect on cercariae and, the effect, or lack thereof, appear to be species specific. For example, several studies on echinostome cercariae indicate that atrazine affects the survival of cercariae after 14 hours of exposure at relatively high (>100μg/L) concentrations (Griggs and Belden 2008; Koprivnikar et al. 2006b; Rohr et al. 2008a). Although tadpoles tend to be immunocompromised when exposed to pesticide mixtures of which atrazine is the main component (Christin et al. 2003; Gendron et al. 2003), when both the cercariae and tadpoles are exposed, higher infections (i.e., intensities and prevalences) are not observed (Koprivnikar et al. 2007b); however, interactive effects of atrazine and parasitism begin to emerge (Koprivnikar 2010). Koprivnikar et al. (2006b) explored the effects of atrazine (200 μg/L) on the survival of field-collected snails releasing cercariae of 3 trematode species and observed extreme variation in the longevity of the 3 trematode species. Atrazine only affected the longevity of 1 of the 3 trematode species and they found the effects to be highly species specific. Despite these findings, it is unclear how effects on cercarial survival translate into infectivity. In contrast to these previous experiments on trematode species that have free-swimming cercariae, *H. eccentricus* has cystophorous cercariae which can survive for weeks in the external aquatic environment (Shostak and Esch 1990). Although we did not test the effects of atrazine on the longevity of cercariae, we did test the effects of atrazine on infectivity of *H. eccentricus* in ostracods. Cercariae produced from snails in control and atrazine treatment groups did not exhibit any significant differences in infectivity to ostracods, suggesting there were no carry over or

epigenetic effects of atrazine exposure to miracidia or other intramolluscan trematode stages. Additionally, the atrazine concentration that ostracods were exposed to did not significantly affect prevalence. The infection mechanism of cystophorous cercariae in *Halipegus* spp. involves injecting the body of the cercaria into the ostracod through a delivery tube (Bolek et al. 2010; Zelmer and Esch 1998b). Atrazine appears to have no direct effect on this process.

As for production of cercariae, we did not detect any direct effects of atrazine on the daily production of cercariae. Although Koprivnikar and Walker (2011) observed generally fewer echinostome cercariae being produced in naturally infected snails (*Stagnicola elodes*) exposed to solvent control and desethylatrazine (a primary metabolite of atrazine) treatments, the effect was not significant.

After cercariae enter the appropriate second intermediate host, they form a cyst, or metacercaria. Most studies have focused on the ability of cercariae to establish infections in second intermediate larval amphibian hosts, but none have assessed the infectivity of those cysts to the subsequent host. Although we did not directly expose metacercariae of *H. eccentricus* to odonates or amphibians to verify infections, it has been established that metacercariae of *Halipegus* species including *H. eccentricus* are developmentally incapable of infecting amphibian hosts until they pinch off their skirt-like bladder (Bolek et al. 2010; Zelmer and Esch 1998a). Thus, our measure of infectivity refers to the developmental capability of infecting the next host. Our results indicate that metacercarial infectivity is significantly reduced at 3 and 30 μg/L atrazine relative to controls. It is possible that, with more time, all the metacercariae would have eventually become infective. However, if infected ostracods were ingested prior to development, the

life cycle would not continue (Zelmer and Esch 1998a). Thus, extending the number of days it takes *H. eccentricus* to become infective will undoubtedly result in reduced transmission.

In addition to concerns about trematode transmission, it is also important to consider the additional stress of contaminants on host-parasite interactions (Marcogliese and Pietrock 2011; Rohr and McCoy 2010; Sih et al. 2004). Our results clearly demonstrate that infections by *H. eccentricus* exhibit a biologically negative effect on snail (*P. acuta*) host survival, reducing the longevity of atrazine control snails by 32.4%. In a review, Gustafson et al. (2015) suggest atrazine exhibits negligible direct effects on snail survival at environmentally-relevant concentrations. Although atrazine alone did not have a significant effect on snail survival, atrazine interacted synergistically with parasitism and reduced the longevity of snails exposed to atrazine (3 and 30 μg/L) by 37.3% and 50.8%, respectively. It appears that the additional stress of atrazine enhances the negative effects of *H. eccentricus* on host health. As a result, the duration of cercarial shedding was truncated and the net benefit to the parasite was reduced.

No other study has tested the interactive effects of atrazine and parasitism on the survival of laboratory-infected snails. Most studies exposed either uninfected snails or free-swimming miracidia/cercariae to atrazine. However, for the duration that snails and trematodes co-occur in a water body, it is likely that these potential hosts and parasites would be concurrently exposed to similar aqueous atrazine concentrations. Interestingly, Koprivnikar and Walker (2011) tested the interactive effects of desethylatrazine and parasitism on wild-caught snails (*S. elodes*). Although the pesticide exposure history, duration of trematode infections, and the ages of the snails used in their study were

unknown, they found a slight, but significant, reduction in snail survival when snails were infected with gymnocephalus type cercariae and subsequently exposed to a desethylatrazine concentration of 0.33 μg/L, which is a much lower concentration than used in this study. In contrast, patent echinostome infections did not act synergistically with desethylatrazine to reduce snail survival. In fact, snails with prepatent echinostome infections tended to live longer than uninfected snails in the control, solvent control, and desethylatrazine treatments. This observation could partially explain the increase of echinostomes in agricultural ponds and the absence of other trematode species (King et al. 2007; King et al. 2010; Marcogliese et al. 2009). We used laboratory-raised snails and followed the entire life of each infected snail (and uninfected reference snail) and thus are unable to directly compare our results to Koprivnikar and Walker (2011). However, our observation of multiple stressors acting together to reduce snail fitness does not appear to be unique (reviewed by Morley 2010). For example, studies on atrazine combined with predation cues (Sandland and Carmosini 2006) and infected snails given low food supplies (Krist et al. 2004) both demonstrate interactive effects on snail fitness.

In contrast to snails, atrazine had a direct negative effect on ostracod survival. Currently, there are no known studies testing the effects of atrazine on any species in the genus *Cypridopsis*. Short (2-3 day) acute atrazine toxicity tests have observed LC50s (i.e., lethal concentrations to 50% of the sample population) to other microcrustaceans as low as 15 μg/L (Detenbeck et al. 1996). However, LC50s typically occur at much higher concentrations (12 - 72 mg/L; e.g., Wan et al. 2006). Our chronic 22 day exposure to 30 μg/L atrazine reduced ostracod survival to 45.5% compared to control ostracods. The mechanism of this toxicity is currently unknown and could be driven by direct toxicity to

ostracods. Alternatively, given that atrazine is an herbicide, atrazine has a high propensity to affect plant food sources and could have indirectly affected ostracod survival via effects on food quality. Interestingly, when ostracods were simultaneously exposed to cercariae and atrazine, the negative effects of atrazine on ostracod survival were ameliorated. Our study is not the first to observe increased longevity of *Halipegus*-exposed ostracods. Parasitism by *H. eccentricus* may increase the longevity of ostracods as has been previously suggested by Zelmer and Esch (1998c) who observed significantly higher survival of ostracods (*Cypridopsis* sp.) when exposed to *H. occidualis*. As for reducing toxicity, metacercariae of *H. eccentricus* inhabit the majority of the space inside the ostracod and the toxicity to ostracods could potentially be reduced via chemical metabolism by the unencysted metacercaria. It is also possible that metacercariae bioaccumulate atrazine from ostracod hosts as in other contaminant-host-parasite systems (Sures 2004); however, currently there is no evidence suggesting trematodes absorb atrazine.

Although it appears that trematodes may exhibit beneficial effects (i.e., increased survival of exposed ostracods), ostracods may be unable to reproduce when infected (Zelmer and Esch 1998c) and it is currently unknown whether ostracods can outlive their infections. Thus, we expect that, even though metacercarial development is reduced or delayed, increased ostracod survival in the presence of atrazine ultimately benefits the worm, not the ostracod.

Conclusions

This study demonstrates that each trematode life stage and each host species responds differently to atrazine exposures. Additionally, throughout a trematode life

cycle, each life stage typically infects a different host and those unique host/parasite relationships can be affected by pesticides. In this study, there were multiple indications of reduced transmission, including reduced production of cercariae and reduced metacercarial infectivity. However, there was no indication of enhanced transmission. Thus, for *H. eccentricus* (and potentially other species), atrazine (and potentially other pesticides) appears to reduce trematode transmission which has important implications for the maintenance of trematode biodiversity in altered ecosystems. This study shows environmental effects on host-parasite interactions should be examined at each stage and provides a testable mechanism to better understand altered trematode transmission in contaminated ecosystems.

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Table 4.1. Atrazine effects on life history parameters of hosts (Snail: *Physa acuta*; Ostracod: *Cypridopsis* sp.) and parasites (*Halipegus eccentricus*). Asterisks indicate significant differences between atrazine treatment groups. Lowercase letters indicate significantly homogenous groups. Net result of infective worms was calculated based on the total cercariae produced and the metacercaria infectivity; the control was standardized to 100%.

Variable	Atrazine (μg/L)		
	0	3	30
Snail experiment			
Trematode prevalence in snails (% ± se)	19.8 ± 8.7	14.4 ± 9.1	10.7 ± 9.4
Uninfected snail survival (mean days ± se)	63.2 ± 2.6	62.4 ± 4.1	68.7 ± 2.4
Infected snail survival (mean days ± se)*	42.7a ± 1.5	39.1ab ± 2.1	33.8b ± 0.7
Days releasing cercariae (mean ± se)*	13.3a ± 1.4	8.0b ± 1.4	5.5b ± 0.6
Cercariae produced per snail (median, mean ± se)*	679a, 792.8 ± 108.2	352ab, 536.3 ± 124.9	152b, 236.9 ± 153.1
Total cercariae produced (grand total)	15,856	8,044	2,369
Ostracod experiment			
Prevalence in ostracods (% ± se)	29.4 ± 4.2	19.5 ± 4.4	20.0 ± 4.5
Parasite-unexposed ostracod survival (% ± se)*	91.7a ± 4.0	93.8a ± 3.5	41.7b ± 7.2
Parasite-exposed ostracod survival (% ± se)	82.6 ± 3.2	85.4 ± 3.6	83.3 ± 3.8
Metacercaria infectivity (% ± se)*	58.3a ± 7.5	18.8b ± 11.3	16.7b ± 10.7
Net result of infective worms	**100.0%**	**16.4%**	**4.3%**

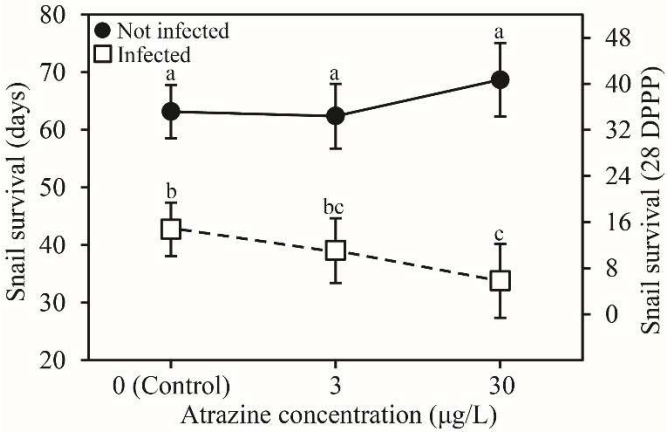

Fig. 4.1. Effects of atrazine treatment on the number of days uninfected and infected snails (host: *Physa acuta*; parasite: *Halipegus eccentricus*) survived (left y-axis). For visualization, we also present the number of days snails survived 28 days post prepatent period (DPPP; right y-axis). Means ± 95% confidence intervals are presented. Lowercase letters indicate significantly homogenous groups based on Tukey's unequal N HSD post hoc tests.

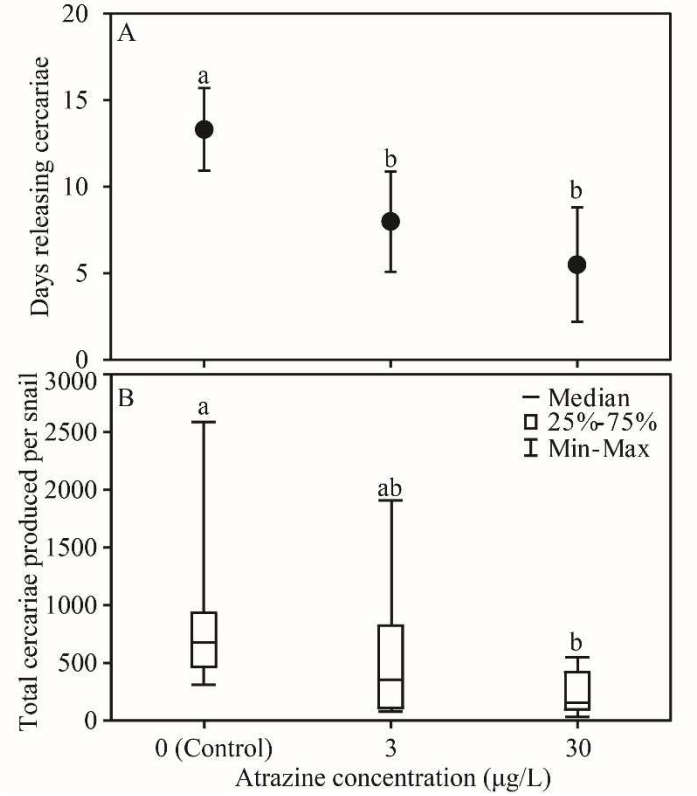

Fig. 4.2. Effects of atrazine treatment on (A) the number of days snails released cercariae, and (B) the total number of cercariae produced throughout each snail's lifetime. (A) Means ± 95% confidence intervals and (B) medians, 25-75% interquartiles, and ranges are presented. Lowercase letters indicate significantly homogenous groups based on (A) Tukey's unequal N HSD post hoc tests or (B) Kruskal-Wallis multiple comparisons of mean ranks z tests.

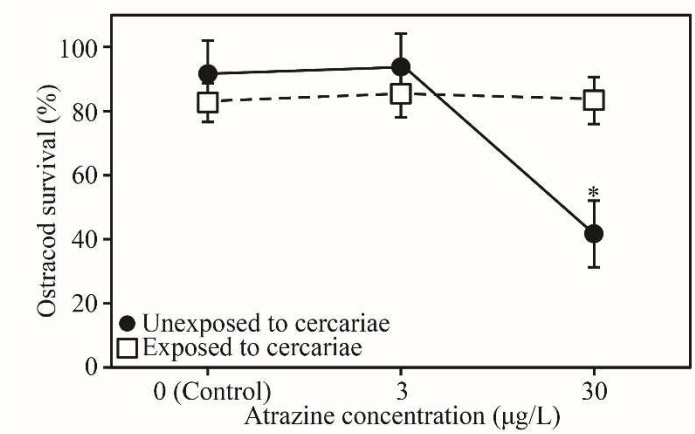

Fig. 4.3. Effects of atrazine treatment and parasite exposure on the survival of ostracods 22 days post exposure with *H. eccentricus*. Means ± 95% confidence intervals are presented. The asterisk indicates the only significantly different group based on Tukey's unequal N HSD post hoc tests.

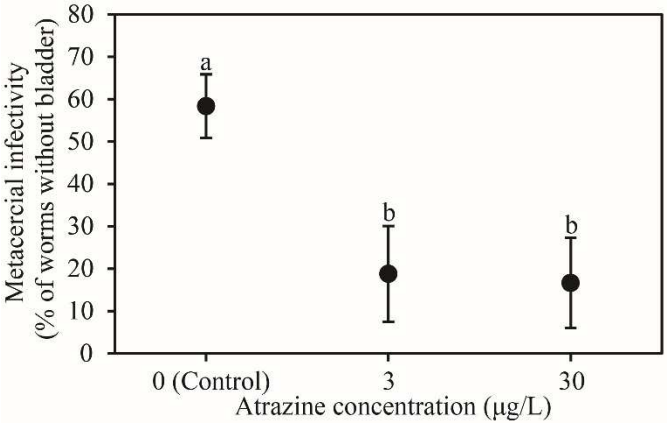

Fig. 4.4. Effects of atrazine treatment on the potential infectivity of 22 day old metacercariae to the next host in the life cycle. Means ± 95% confidence intervals are presented. Lowercase letters indicate significantly homogenous groups based on Tukey's unequal N HSD post hoc tests.

CHAPTER V

DISTINCT SNAIL (*PHYSA*) MORPHOTYPES FROM DIFFERENT HABITATS CONVERGE IN SHELL SHAPE AND SIZE UNDER COMMON GARDEN CONDITIONS

Gustafson, KD, Kensinger BJ, Bolek MG, Luttbeg B (2014) Distinct snail (*Physa*) morphotypes from different habitats converge in shell shape and size under common garden conditions. Evolutionary Ecology Research 16:77-89

This research was original and was primarily conducted by myself and Bart Kensinger under the advisement of our advisors, Matthew Bolek and Barney Luttbeg. This chapter was published in the journal *Evolutionary Ecology Research*.

ABSTRACT

Background: Aspects of snail shell morphology may be plastic or genetically fixed. Even within a single population, environmentally induced shell shape plasticity can lead to unclear species identifications as a result of extreme shape variation. Extrinsic factors, such as predation pressure and stream flow, tend to induce adaptive plastic changes in shell morphology, such as elongate shells with narrow apertures and short-spired shells with wide apertures, respectively. Snail populations from a local stream and adjacent wetland exhibit these distinct morphotypes. **Questions:** Do the snail morphotypes represent a single cryptic species? Are the morphotypes environmentally induced and plastic, or epigenetic? **Organisms:** We captured wild *Physa* snails from either a stream population (low predation, high flow site) or a nearby pond population (high predation, low flow site) in Stillwater, OK, USA. **Predictions:** If distinct snail morphotypes represent a single cryptic species, their phenotypes may be plastic. In this case, raising snail offspring under similar conditions of predation and stream flow will result in one shell shape and size. **Methods:** We reared and maintained snail offspring of both morphotypes in laboratory aquaria, (low water flow, no predation). We measured the shell morphology of wild, of first-generation laboratory, and of second-generation laboratory snails using geometric morphometrics. **Results:** Shell shape and size of wild snails from the two populations were significantly different. After a single generation, however, the shell shape of both populations resembled the wild snails from the pond site (elongate with narrow apertures). Shell size decreased in the first generation, but shell size in the two populations did not fully converge until the second. **Conclusions:** The shape differences are plastic responses to environmental variation. Thus, the two

morphotypes constitute a single snail species (*Physa acuta*). The single generation lag in size convergence suggests there is an epigenetic difference between generations within populations.

INTRODUCTION

Phenotypic plasticity is a major focus of ecological and evolutionary research (West-Eberhard 1989; DeWitt et al., 1998; Pfennig et al., 2010), especially with regard to its adaptive nature (Pigliucci, 2001). The ability of similar genetic backgrounds to produce wildly different phenotypes in different environments has been of notable interest across a diverse array of taxa (e.g., Bradshaw, 1965), including snails (Vermeij, 1995), which are becoming model organisms for the study of plasticity (Brönmark et al., 2011).

Snail shell dimensions, the most easily quantifiable of morphological traits, have historically been considered phylogenetically informative in several publications regarding species identification (e.g., Burch, 1989). However, the ecological conditions of freshwater habitats have been suggested to induce plastic, sometimes adaptive, changes to shell morphology (Vermeij, 1995) and it is becoming increasingly evident that shell plasticity has led to historically unclear species identifications (Minton et al., 2008; Perez & Minton, 2008). For example, snail species with extensive gene flow between populations can have dramatically different phenotypes within populations due to different habitat conditions, which has resulted in the misidentification of species based on morphology (Johnson & Black, 1999). Additionally, local selection pressures can result in cryptic phenotypic plasticity within a single snail population when extreme

intrapopulation morphological variation incorrectly suggests there are multiple species (Dillon et al., 2013).

Historical studies suggest stream size and flow induce dramatic changes in mollusc shell shape (Ortmann, 1920; Ball, 1922; Wiebe, 1926). This hypothesis has been tested several times and, generally, as a plastic response to water current and potential dislodgement, snail foot size and shell aperture width increase (Kitching et al., 1966; Trussell et al., 1993). Although water current undoubtedly has a large impact on shell plasticity, reciprocal transplant experiments suggest water current is not the sole factor affecting snail shell morphology (Etter, 1988). Dillon (2011) notes that differences in water flow are associated with differences in water chemistry, temperature, productivity, and biotic communities. This includes predation, which has also been suggested as a major force affecting snail shell morphology (Palmer, 1979; Trussell, 1996; DeWitt et al., 2000; Langerhans & DeWitt, 2002; Salice & Plautz, 2011). In general, these studies indicate predation cues induce snails to produce thick, elongate shells with narrow apertures.

Snails in the family Physidae are morphologically diverse and molecular data suggest morphologically-based species descriptions have overestimated physid species diversity by 50% (Wethington & Lydeard, 2007). Although several studies have demonstrated shell plasticity in physid snails when they were exposed to different environmental conditions (DeWitt et al., 2000; Langerhans & DeWitt, 2002; Britton & McMahon, 2004), to our knowledge, no study has assessed changes in shell shape and size when distinct morphotypes experience the same ecological conditions.

In this study, we hypothesize that, in general, physid morphotypes are not distinct genetically, but are plastic responses to their environment. To test this hypothesis, we use two distinct *Physa* morphotypes: one from a pond in Teal Ridge Municipal Park and one from an adjacent stream in Babcock Park, Stillwater, OK. Stevison (2013) suggested that these two unique site-specific morphologies are a response to the high density of predatory crayfish at Teal Ridge (more than 40 crayfish per trap) and the low density of predatory crayfish at Babcock Park (0 crayfish per trap but visually observed at the site). However, in his study, he did not consider the effect of water current or other ecological factors on snail morphology. Here, we provide, the first experimental assessment of these two hypotheses by raising these two geographically adjacent, but distinct *Physa* morphotypes in a common garden (with no predators and no water current), and quantifying their morphology for two generations. If predation is the main driver of snail shell divergence between these two populations, we would expect to see the shape of snails previously exposed to predation pressure to converge across generations towards a shape of snails experiencing low predation pressure (Stevison, 2013). In contrast, if water current is the main driver of snail shape, we would expect to see the opposite pattern. It is also possible, given the numerous differences between pond and stream environments, that both processes and/or additional processes are occurring simultaneously, in which case, the shell shape of common garden offspring would be expected to diverge away from both wild population morphotypes and converge towards a novel morphotype.

MATERIALS AND METHODS

Specimen collection and culture

We collected *Physa* snails from a pond at Teal Ridge Municipal Park [a high predation/low flow (HP/LF) site] and a stream in Babcock Park [a low predation/high flow (LP/HF) site] on May 31, 2013 in Stillwater, OK, USA. Teal Ridge (henceforth: HP/LF) is a restored wetland area characterized by ephemeral ponds that remain seasonally inundated the majority of the year; whereas Babcock Park (henceforth: LP/HF) is located along Stillwater Creek. The stream flows from an Army Core of Engineers Dam approximately 10 km up stream. Collection sites are approximately 600m away. However, at its minimum, the pond is only 200m away from the creek and during heavy rains, drains into Stillwater Creek through a man-made drainage. Approximately one hundred snails of unknown ages were collected from each location and placed in one of two respective 5-gallon sterile buckets filled 2/3 full with dechlorinated well water. Ten algal pellets were placed in each bucket as a food source for snails and buckets were aerated in the laboratory overnight. To induce snails to lay eggs, Porcelain, Styrofoam, and plastic containers were placed in each bucket as egg-laying substrates. Egg masses were collected from each substrate the following day. Immediately after egg collection, we preserved 20 wild (generation F0) randomly selected snail individuals from each population in 95% ethanol.

Next, we prepared six 10-gallon tanks filled with 9.8 gallons of dechlorinated tap water and 5 cm of washed gravel for substrate. All tanks were aerated, room temperature was held constant at 25±1°C, and fluorescent lights were set to a 14-10 light-dark cycle. Five egg masses (containing 50-60 eggs each) produced by F0 snails from the HP/LF site

were split into thirds and deposited evenly into 3 of the 10-gallon laboratory tanks. We then repeated this procedure for the other 3 tanks with eggs produced by F0 snails from LP/HF site. In this way we controlled for the genetic structure in each tank, but attempted to maintain some of the variation from each population. Hatched first laboratory generation (F1) snails were feed algae pellets and frozen green leaf lettuce *ad libitum*. Consistent with previous reports (Wethington & Dillon, 1993), F1 snails began laying eggs within 6-7 weeks of hatching.

Seven weeks after hatching, we removed all F1 snails from each tank and left all snail egg masses produced by the F1 individuals intact. We preserved 20 randomly selected F1 snails from each tank in 95% ethanol. Similarly, after an additional 7 weeks, 20 randomly selected second generation (F2) laboratory snails were preserved from each tank as previously described. To assess snail size and shell morphology, we photographed all preserved F0, F1, and F2 snails. Briefly, fixed snails were individually placed ventral side up next to a ruler with the apex away from the individual taking the photograph (Fig. 5.1a). Digital images were taken with a Canon G11 Powershot digital camera attached to an Olympus SZ61 tri-head dissecting microscope. All snails were deposited as voucher specimens in the Museum of Zoology: Mollusk Division, University of Michigan (UMMZ accession numbers: UMMZ304402-UMMZ304407).

Molecular and morphological species identification

We sequenced the cytochrome oxidase *c* subunit I (COI) mitochondrial gene for 3 individuals from each population. All DNA extractions were performed by grinding frozen (-80˚F) soft tissue from snail bodies and using E.Z.N.A.® Insect D.N.A. Kits (Omega BioTek). We used the following primer pair: LCO1490 (forward) 5'- GGT CAA

CAA ATC ATA AAG ATA TTG G -3' and HCO2198 (reverse) 5'- TAA ACT TCA GGG TGA CCA AAA AAT CA -3'. We performed PCRs using an initial denaturing of 94˚F for 3 min, followed by 35 cycles of denaturation at 94˚F for 30 sec, annealing at 47˚F for 1 min, and extension at 72˚F for 1 min. We used one final hold of 72˚F for 7 min. PCR products were gel-purified and cleaned with the Wizard® SV Gel and PCR Clean-Up System (Promega). Sequencing was performed in both directions for all sequences at the Core Facility of Oklahoma State University on an ABI7000 sequencing analyzer. We reverse complimented and aligned sequences using CLC Main Workbench 6.8.2, then edited alignments by hand. Sequences were subsequently deposited in GenBank (Accession numbers: KJ769123-KJ769128). DNA sequences were compared to other sequences in GenBank using BLAST (Morgulis et al., 2008) which utilized program BLASTN 2.2.29 (Zhang et al., 2000). Additionally, snails were identified by penial complex according to Wethington (2004) and Wethington & Lydeard (2007).

Shape and size analyses

We used geometric morphometric methods to assess snail shell morphology (Rohlf & Marcus, 1993; Zelditch et al., 2004). We digitized 22 landmarks (Fig. 5.1) on digital images of each specimen (N = 260) using tpsDig2 (Rohlf, 2013). Snails that lacked homologous landmarks (i.e., discrete and easily recognizable endpoints) or semilandmarks (i.e., not a discrete endpoint; treated here as equidistant points along curves or whorls) because of broken shells (typically broken apertures and apexes) were removed from the analyses (see Zelditch et al., 2004 for landmark discussion), resulting in 128 HP/LF snails (F0: 20, F1: 57, F2: 51) and 128 LP/HF snails (F0: 19, F1: 55, F2: 54). We used tpsRelw (Rohlf, 2010) to perform generalized least-squares Procrustes

superimposition (i.e., rotation, translation, and scaling to remove positional effects; Bookstein, 1991) to obtain shape variables (partial and relative warps) for further analyses.

Relative warp (RW) axes (i.e., axes that summarize the variation in shape among the specimens) were used to test population source and generation effects on snail shell morphology using a multivariate analysis of covariance (MANCOVA). Additionally, we calculated a divergence vector (DV), according to Langerhans (2009), as a measure of directional differences in shape across full shape space (i.e., all RWs). Briefly, we performed a principal components analysis (PCA) of the sums of squares and cross-products matrix of the generation term from the MANCOVA to derive an eigenvector of divergence (Langerhans, 2009). The DV describes linear combinations of dependent variables exhibiting the greatest differences between generations (within populations) in Euclidean space and avoids distortion of shape space. An ANCOVA was used to test for significant shape divergence between generations within and between source populations. Centroid size (i.e., the square-root of the sum of squared distances from the landmarks to the centroid of the landmarks) was used as the measure of snail size. To account for geometric morphometric allometry, centroid was included as a covariate in all shape analyses. Additionally, an ANOVA of centroid was used to test for significant generational differences in size within and between populations. To allow for easier interpretation, we also calculated snail shell length, shell width, aperture length, aperture width, spire length, and spire angle using tpsDig and assessed the correlation of these traditional snail shell metrics with RW axes, DV, and centroid. We used tpsRegr (Rohlf, 2011) to visualize shape differences relative to DV scores.

RESULTS

Molecular and morphological species identification

The lengths of the 6 COI gene sequences ranged from 615 to 684 bp. For all 6 sequences, the BLAST search resulted in 72 hits for *Physella acuta* (≈ *Physa acuta*; Wethington & Lydeard 2007) and 2 hits for *Physella virgata* (recently synonymized with *Physa acuta*; Wethington & Lydeard 2007). Additionally, for all sequences, at least the first 20 BLAST results for each sequence had a 98-99% identity score for *Physella acuta* and/or *Physella virgata*. The top BLAST results, in all cases, included the sequence AY651170.1 (*Physella virgata*) or KF737951.1 (*Physella acuta*) (avg. max score: 1154.67 ± 37.55; avg. max identity 98.83% ± 0.41%; avg. E-value: 0.0 ± 0). Analyses based on penial morphology were consistent with molecular sequence data. Each snail had the same overall penial complex indicative of *Physa acuta*, including a preputial gland and one-part, non-glandular, muscular, penial sheath (Wethington 2004; Wethington & Lydeard 2007).

Shape and size analyses

The relative warp (RW) analysis resulted in 9 RW axes each accounting for more than 1% of the overall shape variation. Of those, the first 2 RW axes accounted for 70.11% of the shape variation (Fig. 5.2) and RW axes 1-4 (81.75% cumulative shape variation) were the only axes to each account for more than 5% of shape variation (Table 5.1). RW axis 1 was significantly and primarily related to measures of snail shape, including various spire and aperture width-length ratios and was weakly related to snail length and width measurements by themselves (Table 5.1). We interpret the range of RW axis 1 as a change in snail shell shape from shells with relatively wide apertures and

relatively short spires to shells with relatively narrow aperture widths and relatively long spires. RW axis 2 was also significantly and primarily related to snail shape, including spire length ratios, aperture-length to shell-length ratio, and spire angle (Table 5.1). We interpret RW axis 2 as a change in snail shell shape from shells with relatively long apertures and spires to more compact shells (i.e., those with relatively short aperture and spire lengths). Additionally, spire angle decreased with increasing values on both RW axes.

For snails reared in laboratory tanks (i.e., F1 and F2), nested ANOVA results indicate that tank, nested within source, did not have a significant effect on shape ($F_{4,210}$ = 0.494, P = 0.74). Additionally, there was no significant effect of tank when nested within source x generation ($F_{9,205}$ =1.03, P = 0.41). Thus, tank was dropped from the analysis and snails from the same source and same generation were pooled for further analyses. Size was significantly related to shape (Table 5.2), indicating the presence of geometric morphometric allometry. Thus, size was included as a covariate in all subsequent analyses. MANCOVA results indicate that there were significant shape differences between source populations, generations, and a significant source x generation interaction (Table 5.2 & Fig. 5.3).

The interpretation of DV is similar to the interpretation of RW axis 1 where snail shells with negative DV scores had relatively wide apertures, relatively short spires, and wide spire angles; snail shells with high DV scores had relatively narrow apertures, long spires, and narrow spire angles (Table 5.1 & Fig. 5.4). However, compared to RW axis 1, DV had lower correlation coefficients with specific variables as a result of being a measure over full shape space (i.e., over all RW axes). ANCOVA results for DV were

congruent with our MANCOVA results for RW axes (Table 5.2). However, the DV analysis made multiple comparisons readily apparent. Results of the ANCOVA indicated that wild (F0) stream (LP/HF) and pond (HP/LF) snails had significantly ($P < 0.001$) different shapes (Figs. 3 & 4). However, there were no significant shape differences between source populations within the F1 generation ($P = 0.65$) or the F2 generation ($P = 1.0$) (Figs. 3 & 4a). In a multiple-comparison of means, the LP/HF F0 generation of snails was the only group to be significantly different from all the other groups (Fig. 5.4a) and was the only group with a centroid and 95% confidence intervals that did not cross the intercept of either major RW axis (Fig. 5.3d). After a single generation, the shape from both populations converged (Figs. 3b & 4a). Finally, the shape of both F2 generation snails converged in the opposite direction of the LP/HF F0 snails (Fig. 5.4a). The shell shapes of F2 generation snails from both populations were nearly identical to and not significantly different from HP/LF F0 snails (Figs. 3d & 4a). Additionally, HP/LF snails (F0, F1, and F2) did not significantly change shape throughout the experiment (Fig. 5.4a). DV scores ranged from -13.14 to 8.73 and a gradient in shell shape (from snails with wide apertures, short spires, and wide spire angles to snails with narrow apertures, tall spires, and small spire angles) could be visualized over the observed range (Fig. 5.4b).

Size (centroid) showed similar patterns to shape where size was significantly related to generation ($F_{2,250} = 192.43$, $P < 0.001$), population source ($F_{1,250} = 159.69$, $P < 0.001$), and a generation x source interaction ($F_{2,250} = 54.24$, $P < 0.001$). All statistical relationships remained significant when the wild-caught F0 generation snails (of unknown age), were removed from the analysis. Overall, wild HP/LF snails were much

larger than wild LP/HF snails (Mean shell length±SD in mm; HP/LF F0: 10.38±1.47; LP/HF F0: 6.96±1.17). Within each population, size significantly decreased in the first generation (HP/LF F1: 7.64±0.95; LP/HF F1: 6.17±0.72) until the sizes of snails from both populations converged in the F2 generation (HP/LF F2: 5.46±0.81; LP/HF F2: 5.53±0.75) (Fig. 5.5). Centroid was significantly and strongly correlated to length and width variables (Table 5.1), all of which showed the same patterns as centroid (data not shown). Centroid was also negatively correlated to spire angle, suggesting larger individuals had smaller spire angles as indicated by the strong relationship with the spire-length to spire-angle ratio (Table 5.1).

DISCUSSION

Our work indicates that although offspring of wild snails from both populations became smaller, they both converged on a single phenotype. In addition to the molecular and penile data, the complete convergence of both size and shape in these two populations suggests that these two morphotypes are indeed the same species, *Physa acuta*, and the phenotypic differences are plastic responses to environmental variation. We suggest that this pattern may be common in freshwater snails and our study supports the overestimation of physid species based on shell characteristics alone (Wethington & Lydeard, 2007).

The most notable result of shape, which rapidly converged, is that both populations converged on the wild pond (HP/LF) morphotype. Although offspring of snails from the pond (HP/LF) dramatically decreased in size compared to offspring of snails from the stream (LP/HF) habitat, it was the offspring of stream snails (LP/HF) that

changed shape and ultimately converged with the shape of F0, F1, and F2 pond (HP/LF) snails. Several studies have demonstrated a plastic response of snail shell morphology to predation (Palmer, 1979; Trussell, 1996; DeWitt et al., 2000; Langerhans & DeWitt, 2002; Salice & Plautz, 2011). For these two *P. acuta* populations specifically (elongate shells with narrow apertures vs. short-spired shells with wide apertures), Stevison (2013) hypothesized that the unique site-specific morphologies were a response to the high density of predatory crayfish at the Teal Ridge pond (HP/LF) and the low density of predatory crayfish at Stillwater Creek (LP/HF). This hypothesis predicts that the lack of predators in the laboratory would release any energetically costly predator-induced morphologies, resulting in a shell shape shift towards the stream (LP/HF) morphotype (short-spired shell with wide aperture) or resulting in a novel morphotype. This was not the case and, although there are many potentially different environmental characteristics between ponds and streams, these results are congruent with the hypothesis that water current is a major factor affecting molluscan shape. The water current in Stillwater Creek is relatively high compared to the standing water in the Teal Ridge pond, and as an induced plastic response, foot size and aperture width may increase for stream (LP/HF) snails to resist dislodgement (Kitching et al., 1966; Etter, 1988; Trussell et al., 1993). It is also possible that a predator-defended phenotype was retained throughout generations in the pond (HP/LF) snails, even in the absence of predatory cues. Similarly, in the absence of water flow, the offspring of stream (LP/HF) snails could have converted to a "default" predator-defended phenotype. In other words, predation could be the predominant ultimate cause of shell morphology, whereas water current could simply be a proximate cause. However, the shape of the wild pond snails (HP/LF) may not have been a specific

response to predation at all, and the resultant shape of wild pond snails (HP/LF) and laboratory snails may simply be a "default" developmental morphotype of *P. acuta* snails in standing-water environments.

Whereas shape appears to be plastic and converged on the pond morphotype (HP/LF) after a single generation, snail size did not converge until the F2 generation. We hypothesize that the reduction in snail size was not a response to selection or any specific genetic differences between populations and generations but a result of early maturation. For example, Clampitt (1970) observed dramatic size reductions and early maturation in two *Physa* species under laboratory conditions where snails tended to mature and reproduce well within two months. Similarly, Wethington & Dillon (1993) observed laboratory-reared *Physa* snails reproducing around 6-8 weeks after hatching. DeWitt (1955) suggested the lack of fluctuating environmental variables (i.e., temperature, water level, parasites, dissolved oxygen, and food) allows laboratory snails the opportunity to successfully develop and reproduce, whereas wild snails would be less likely to experience these conditions. Thus, maturation at a smaller size may simply be a result of optimal habitat conditions and the allotment of resources from somatic growth to reproduction. Although both populations became smaller, there was a single generation lag in size convergence which did not occur for shape. This single generation lag in size convergence may be a maternal effect or some other epigenetic difference between generations. An obvious difference between the early environments of the F1 and F2 generation snails was the egg sac itself. The F1 snails were reared from wild snails that produced eggs sacs that could potentially contain hormonal or nutritional differences depending on the nutritional, environmental, or developmental state of the functional

mother. The other major difference between generations was the age and maturity of the bacterial community in each tank. The different sizes of snails between generations may be a result of F2 generation snails being exposed to an older and more stable microbe fauna; however, we could only speculate as to the mechanism for this possible relationship because it has not been studied.

In conclusion, we set out to assess whether the morphological variation between these two geographically adjacent but distinct morphotypes was a result of phenotypic plasticity or genetics. Both pond (HP/LF) and stream (LP/HF) snails are a single species with a large amount of phenotypic plasticity in shell morphology. These snails converged with the shape of pond (HP/LF) snails after only a single generation. Although snails from both populations became smaller, there was a single generation lag in size convergence, suggesting either a maternal or other epigenetic difference between generations, but not a classical genetic difference between populations. Although we cannot rule out other environmental factors being involved in the shell shapes expressed in our laboratory-reared snails, our results suggest that water current (or lack of current and/or associated correlates) has a large impact on snail shell morphology.

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Table 5.1. Significant ($P < 0.05$) Bonferroni-corrected Pearson's R^2 correlations for shape (RW: relative warp axes accounting for >5% of shape variance; DV: divergence vector) and size (centroid) with traditional snail morphological metrics. R^2 values greater than 0.5 are in bold. Non-significant correlations are not presented (--).

	Shape					Size
Variable	RW1	RW2	RW3	RW4	DV	Centroid
shell length (sl)	--	--	--	0.07	--	**0.99**
shell width (sw)	--	--	--		--	**0.97**
aperture length (al)	-0.05	--	--	0.06	--	**0.97**
aperture width (aw)	-0.13	--	--	0.06	-0.07	**0.87**
spire length (spl)	--	0.15	--	--	--	**0.75**
spire angle (angle)	-0.25	-0.27	--	--	-0.27	-0.11
sw:sl ratio	-0.20	-0.27	-0.25	-0.04	-0.24	--
al:sl ratio	-0.38	**-0.52**	--	--	-0.32	--
aw:sl ratio	**-0.63**	--	-0.19	--	-0.48	--
sw:al ratio	--	--	-0.47	--	--	--
aw:sw ratio	-0.40	0.24	--	0.05	-0.24	--
aw:al ratio	-0.31	0.26	-0.26	--	-0.22	--
spl:sl	0.44	0.29	--	-0.08	0.26	--
spl:sw	0.43	0.34	--	--	0.30	--
spl:al	0.45	0.37	--	--	0.29	--
spl:aw	**0.69**	0.07	--	-0.06	0.46	--
spl:angle	0.08	0.18	--	--	0.08	**0.65**

Table 5.2. Multivariate analysis of covariance (MANCOVA) examining shape variation (relative warps) and ANCOVA examining shape variation described by the divergence vector.

Predictor variable	MANCOVA				ANCOVA		
	Wilk's λ	F	df	P	F	df	P
Centroid (size)	0.61	3	40, 210	<0.001	4.51	1, 250	0.035
Population	0.58	4	40, 210	<0.001	17.78	1, 250	<0.001
Generation	0.33	4	80, 420	<0.001	21.9	2, 250	<0.001
Pop x Gen	0.3	4	80, 420	<0.001	13.35	2, 250	<0.001

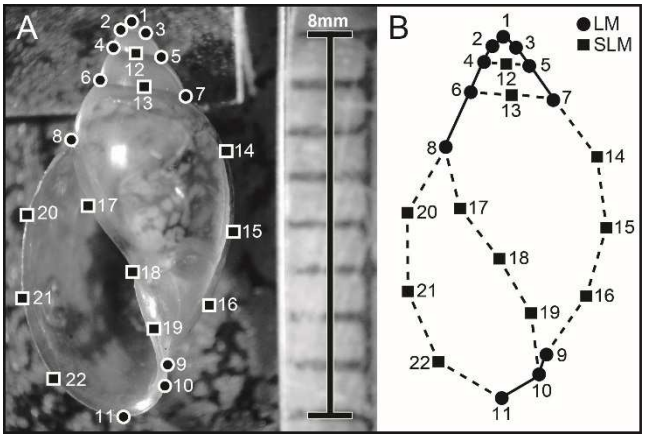

Fig. 5.1. Landmark locations for *Physa acuta* snails on (A) a typical photograph and (B) the consensus snail (i.e., the average shape of all snails). Homologous landmarks (LM; 1-11) are represented by circles. Sliding landmarks (SLM; 12-22) are represented by squares. Dashed lines indicate connections with sliding landmarks and solid lines were added for visualization. Scale bar = 8.0 mm.

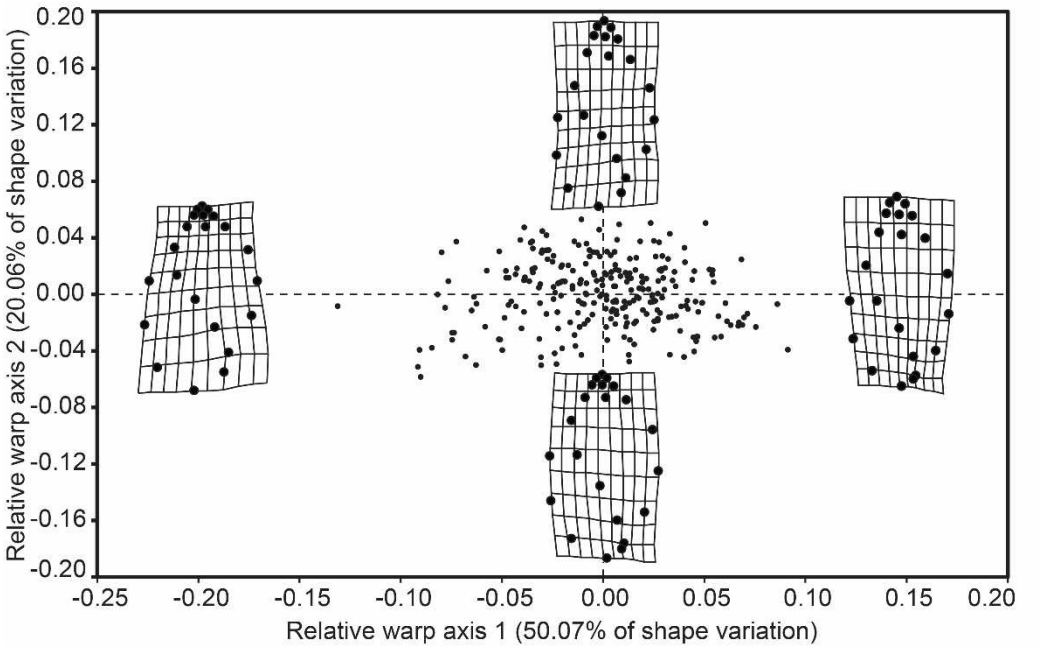

Fig. 5.2. Shape variation of all *Physa acuta* snails (i.e., wild and laboratory-reared) from HP/LF and LP/HF. Overlaid thin-plate spline deformation grids are models of snail phenotypes on the extreme ends of the relative warp axes. Values for the models along the x-axis (y value = 0) are -0.15 and 0.10. Values for models along the y-axis (x value = 0) are 0.06 and -0.06.

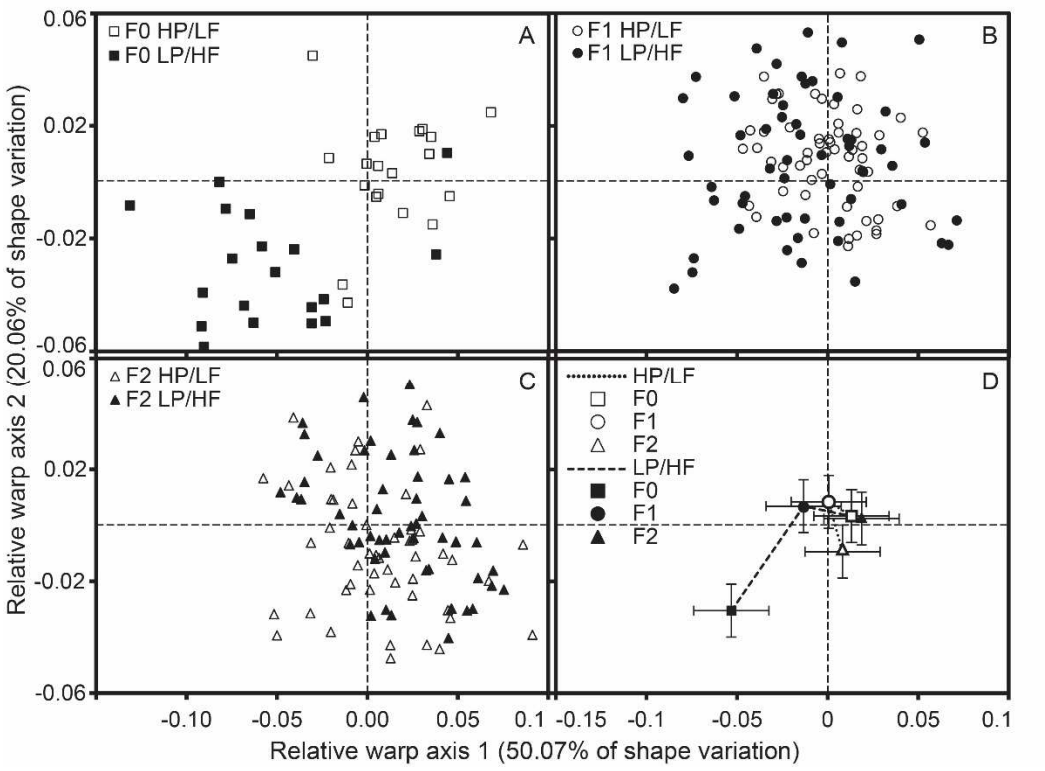

Fig. 5.3. Shape variation of (A) wild (F0) *Physa acuta* snails, (B) first generation (F1) laboratory-reared snails, and (C) second generation (F2) laboratory-reared snails from HP/LF and LP/HF. (D) Combined data with population centroids (±95% confidence intervals) display generational shape changes for each source population.

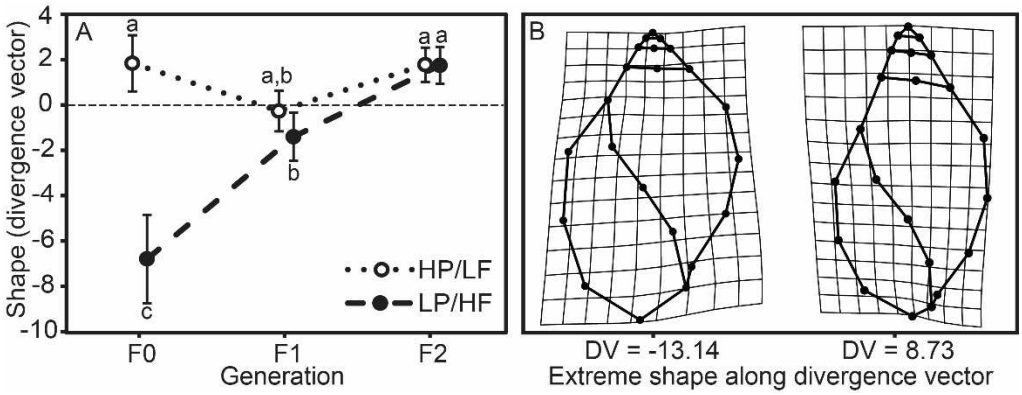

Fig. 5.4. Shape [divergence vector (DV)] convergence (A) of *Physa acuta* snails from HP/LF and LP/HF over full shape space. Generation F0: wild snails; F1: first-generation laboratory-reared snails; F2: second-generation laboratory-reared snails. Means with 95% confidence intervals are presented (letters indicate significantly homogenous groups based on Bonferroni post-hoc tests). Thin-plate spline deformation grids (B) represent extreme shape variation along the DV (i.e., y-axis of Fig. 5.4a) over the observed range. Lines between landmarks are displayed for visual purposes only.

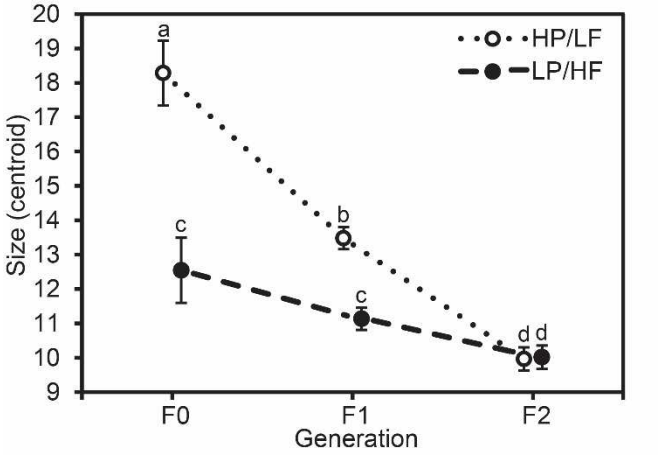

Fig. 5.5. Size (centroid) convergence of *Physa acuta* snails from HP/LF and LP/HF through multiple generations. Generation F0: wild snails; F1: first-generation laboratory-reared snails; F2: second-generation laboratory-reared snails. Means with 95% confidence intervals are presented (letters indicate significantly homogenous groups based on Bonferroni post-hoc tests).

CHAPTER VI

EFFECTS OF TREMATODE PARASITISM ON THE SHELL MORPHOLOGY OF SNAILS FROM FLOW AND NONFLOW ENVIRONMENTS

Gustafson, KD and MG Bolek. 2016. Effects of trematode parasitism on the shell morphology of snails from flow and nonflow environments. Journal of Morphology 277:316–325. doi: 10.1002/jmor.20497

This research was original and was conducted under the advisement of my advisor, Matthew Bolek. This chapter has been published in *Journal of Morphology*.

ABSTRACT

The primary function of the gastropod shell is protection. However, shells that function well in one environment may be maladaptive in another. Upon infection, the snail shell protects internal parasites and it is to the parasite's advantage to optimize, or not interfere with, shell functionality. However, parasites, particularly trematodes, are often pathogenic and it is not clear if parasitism will induce environment-dependent or -independent changes to gastropod shells. We conducted a field study and a complementary laboratory experiment to examine the effects of trematode parasitism on shell characteristics (shape, size, and crush resistance) of *Physa acuta* snails in flow and non-flow environments using geometric morphometrics and crush assays. Field results indicate wetland (nonflow) snails had large, crush resistant shells with narrow apertures and tall spires. In contrast, stream (flow) snails had small, weak shells with wide apertures and short spires. Parasitism had no apparent effect on the crush resistance of wetland snails but significantly reduced the crush resistance of stream snails. Parasitism had no significant effect on overall shell shape in stream or wetland snails. Similar to the results of our field study, nonflow tank snails had significantly more crush resistant shells than flow tank snails. Additionally, the shapes of flow and nonflow tank snails significantly differed where nonflow tank snails resembled wetland snails and flow tank snails resembled stream snails. For laboratory snails, parasitism reduced crush resistance regardless of flow/nonflow treatment. Our results demonstrate that habitat and/or flow treatment was the primary factor affecting *P. acuta* shell morphology and that trematode parasitism played a secondary role.

INTRODUCTION

Although gastropods utilize many physiological and behavioral mechanisms to survive (Dewitt et al., 1999; Townsend and McCarthy, 1980), it is ultimately shell plasticity that allows snails to rapidly adapt to complex, continuously fluctuating environments (DeWitt et al., 2000). Shells are functional structures with the primary purpose of protection (Vermeij, 1995). For freshwater snails, shell size, strength, and shape are traits that allow them to withstand predation and survive in a diverse array of habitats (Vermeij and Covich, 1978). Although having large thick shells has many benefits with regards to resisting predators (Brönmark et al., 2011; Osenberg and Mittelbach, 1989), locomotion and buoyancy are compromised with increasing size (Vermeij, 1973). Predator-defended shells are particularly maladaptive in flowing environments where snails can be dislodged from water forces which exert greater effects on larger, rather than smaller, objects (Denny, 1999; Denny et al., 1985; Trussell et al., 1993).

With any morphological trait, the expressed phenotype is a result of the interactions of the genotype and environment (Pfennig et al., 2010; Pigliucci, 2001; West-Eberhard, 1989). However, gastropods and their parasitic trematodes are so intimately linked in physical space and over evolutionary time that their genomes are essentially non-independent (Lockyer et al., 2004). Thus, it is important to consider the additional effect of trematodes on gastropod shell plasticity. Trematodes are considered "body snatchers" because of their unique ability to infect snails and divert host nutrients to the production of their own clonal parasites (Lafferty and Kuris, 2009). By feeding on gonadal tissue or by interfering with endocrine signaling, parasitic castration causes

snails to cease reproduction and, without the ability to reproduce, the morphological adaptations of the host snail becomes an extended phenotype of the worm (Dawkins, 1999). In essence, the shell protects the trematode and it is within that shell that the trematode must survive to continue its life cycle (Shoop, 1988).

Several studies have identified parasite-induced changes in shell size (i.e., reduced growth or gigantism; e.g., Mouritsen and Thomas Jensen, 1994; Rothschild, 1936; Wilson and Denison, 1980) and/or impacts on shell shape and ornamentation (Hay et al., 2005; Krist, 2000; Lagrue et al., 2007; Żbikowska and Żbikowski, 2005). It has been shown that shells that function well in one environment may be maladaptive in another; however, few studies have addressed parasite-induced effects on shell shape and function in multiple environments (Thieltges et al., 2009). For freshwater snails, wetlands and streams represent two habitats that require different shell morphologies for optimal snail fitness. For example, a large, crush-resistant shell may be well-suited for resisting predation (Brönmark et al., 2011; DeWitt et al., 2000; Langerhans and DeWitt, 2002; Palmer, 1979; Salice and Plautz, 2011; Vermeij and Covich, 1978) but would be a burden in a stream environment where a small, light shell can better resist wave action and dislodgement (Etter, 1988; Kitching et al., 1966; Trussell et al., 1993). Although trematode parasitism has been shown to alter multiple aspects of shell morphology, no study has addressed how trematodes affect multiple measures of shell morphology (size, crush resistance, and shape) in distinct environments where shells must function differently to maximize snail fitness. As a result, there is a major gap in our understanding of adaptive shell plasticity. Although many species of trematodes are thought to be pathogenic, keeping the gastropod host alive ultimately increases

transmission (Gustafson and Bolek, 2015). Thus, it would be to the parasite's advantage to facilitate, or at least not interfere with, shell plasticity. In contrast, trematode parasitism could cause gastropods to express specific morphological changes which could be adaptive or maladaptive in certain environments. Parasitism could also negatively impact snail shell morphology as a result of pathology. Alternatively, trematode infections could exhibit no effects on shell morphology.

To address these hypotheses, we conducted a field study and complementary laboratory experiment to address the effects of trematode parasitism on functional shell characteristics (size, crush resistance, and shape) of *Physa acuta* snails in flow and non-flow environments. We used geometric morphometrics to measure snail size and shape; additionally, we used crush assays to calculate the force required to crush snail shells. For the field study, we used naturally infected and non-infected snails collected from wetlands (i.e., nonflow) and streams (i.e., flow). Given the many differences between wetlands and streams, in addition to (or correlated with) water flow, we conducted a complementary laboratory experiment to better isolate the effects of parasitism and water flow using experimentally-infected and non-infected snails and flow and nonflow tanks.

MATERIALS AND METHODS

Field Study

Throughout June and July of 2014, 4,183 *Physa* snails were collected from 4 wetlands and 4 headwater streams in Payne County, Oklahoma (Table 6.1). Each time we visited a site, we sampled snails for 2 hr duration. During each survey, all observed snails were collected by hand, placed in 19 liter buckets containing 11 liters of water from each

site, and transferred to Oklahoma State University for identification. *Physa* snails from this region have previously been identified as *Physa acuta* (Draparnaud, 1805) based on mtDNA sequences and penile morphology (Gustafson et al., 2014). The penile morphology of 10 *Physa* snails from each location was consistent with *Physa acuta* (Wethington and Lydeard, 2007). The shell length of each snail was measured with a hand-held caliper. Average snail length was 8.8 ± 2.5 (SD) mm with a range from 4.25 to 18 mm. Snails were placed in individual 15 mL wells containing 10 mL of dechlorinated water on a 6-well plate. Snail wells were observed daily (for 3 days) for trematode cercariae with an Olympus SZ51 stereoscope. Water was refreshed daily. Digital photographs were taken of trematode cercariae with an Olympus five megapixel digital camera on an Olympus BX-51 upright research microscope configured for bright-field and differential interference contrast microscopy with plain fluorite objectives. Cercariae were identified to morphotype using keys in Schell (1985).

Trematode Community Structure

For stream- and wetland-collected Oklahoma snails, we calculated prevalence for each cercaria type from each location according to Bush et al. (1997). Additionally, we tested for differences in community structure between wetland and stream snails using a partial canonical correspondence analyses (CCA), implemented in CANOCO 5 (Lepš and Šmilauer, 2003). To account for temporal covariation, we used the collection date (Julian date) as a covariable. Each visit to a site was treated as an individual sample. To standardize among sites, we used the prevalence (i.e., proportion of infected snails) of each cercariae morphotype as measures of abundance. We used the option in CANOCO to down weight the influence of rare morphotypes on the CCA (down weighting gives

weights to species which are related to their prevalences). Permutation tests (999 permutations) were employed to test the significance of habitat type (i.e., wetland or stream) on community composition. To test the effects of specific cercarial types on shell size, crush resistance, and shape (see below), we used nested ANOVAs and treated cercaria type as a nested effect within site. Centroid size was a covariate in crush resistance and shape analyses.

Laboratory experiment

To control for collection environment, any inherited snail morphologies, and because snails and parasites are readily available and their life cycles can be manipulated (Bolek et al., 2010; Gustafson and Bolek, 2015), we collected trematodes and snails from a different location. To obtain trematode eggs for infections, we collected American bullfrogs (*Lithobates catesbeianus* = *Rana catesbeiana*) with dip-nets from Nevens Pond, Keith County, NE (41°12.426'N, 101°24.510'W) in July 2014 and examined their mouths for the trematode, *Halipegus eccentricus*. In the field, 3 gravid *H. eccentricus* worms were removed from the eustachian tubes of a single bullfrog with forceps, placed in a 70 ml plastic jar filled with 30 ml of dechlorinated water, capped, and brought back to the laboratory at Cedar Point Biological Station, Ogallala, NE. At Cedar Point Biological Station, the content of the 70 ml plastic jar was transferred to a petri dish, the worms were shredded with forceps under a dissecting microscope, and the eggs were transferred to a new 70 ml plastic jar filed with 30 ml of dechlorinated water, and the jar was loosely covered. Additionally, we collected 24 adult *Physa* snails from Nevens Pond by hand and placed them in a 19 liter bucket containing 11 liters of wetland water. Field

collected snails were aerated with a battery-powered aerator and trematode eggs were stored in a portable cooler and transported to OSU within 24 hrs of being collected.

For the experiment, we set up 16 37.82 liters aquaria including 8 flow tanks and 8 nonflow tanks. All aquaria contained flow tank inserts (Stream Flowtank™ model 4440B; BioQuip Products, Rancho Dominguez, CA) with 0.5 liters of gravel on top of the flow tank inserts for counterweight and an additional airline tube with an airstone diffuser. In the 8 flow tanks, compressed air was directed to the flowtank inserts which created a counterclockwise current (>30.5cm/sec). In the 8 nonflow tanks, compressed air was directed to the airstone diffuser. Percent dissolved oxygen (DO) was measured daily, using a YSI DO Professional Series Probe (Yellow Springs, OH), in each flow tank to ensure consistent flow. The amount of compressed air directed to the airstone in the nonflow tanks was adjusted to match the DO values in the flowtanks (90-95% DO). This helped to create similar water conditions between treatments except for the circulating current which differed among the 2 treatment groups.

To obtain laboratory cultures of snails from wetland-like or stream-like conditions, the 24 field collected *P. acuta* snails were evenly divided between 2 flow and 2 nonflow tanks. Snail eggs were observed within 24 hrs in each tank. To minimize genetic differences between treatment groups, every day for 7 days, 3 adult field-collected snails from each tank were transferred and allowed to lay eggs in 1 of the other 3 tanks. After 7 days, all 24 field-collected snails were removed and a subset of these snails was crushed. The penile morphology of all crushed *Physa* snails from Nevens Pond was consistent with *Physa acuta* (Wethington and Lydeard, 2007). Laboratory-hatched snails were fed a combination of fresh and frozen green leaf lettuce ad libitum to which a

pinch of TetraMin fish food was added weekly. Water changes (75%) were conducted twice a week (Monday and Friday).

After 3 months of snail growth, we randomly removed 100 snails from each of the 4 culture tanks and pooled them into 2 groups based on treatment (flow or nonflow). Therefore all snails used in this experiment were approximately 3 months in age. Half of the snails from each treatment group (N = 100) were then exposed to 15 trematode eggs containing miracidia of *H. eccentricus* according to Gustafson and Bolek (2015). Briefly, 400 15mL wells (on 6-well plates) were filled with 10 mL of dechlorinated water and 15 *H. eccentricus* eggs were pipetted into each of 200 wells. Two-hundred randomly selected snails sourced from flow tanks and 200 randomly selected snails sourced from nonflow treatments were then systematically placed into the 400 wells, resulting in 100 exposed and 100 non-exposed snails from the flow and nonflow treatment groups. Snails remained in the wells for 72 hours and no snails died during this period. While snails were in well plates, all aquaria were thoroughly cleaned and the culture tanks received new gravel to ensure there were no remaining snail egg masses. Visual inspection of each well containing *H. eccentricus* eggs indicated that each snail ingested the entire dose of trematode eggs.

The shell length of each snail (N = 400) was then measured with a handheld caliper to the nearest 0.25 mm. Average snail length was 5.64 ± 0.83 (SD) with a range from 4 to 8.75 mm. Parasite-exposed snails from flow and nonflow treatments were evenly divided between 4 flow and 4 nonflow treatment tanks, respectively (N = 25 snails/tank). Similarly, non-exposed control snails from flow treatments and nonflow

treatments were evenly divided between 4 flow and 4 nonflow treatment tanks, respectively (N = 25 snails/tank).

The prepatent period of *H. eccentricus* in *Physa* snails ranges from 28-32 days post exposure (Bolek et al., 2010; Gustafson and Bolek, 2015). Thus, snails were maintained in aquaria for 28 days and fed as previously described. Water changes were conducted as previously described and egg masses and dead snails were removed during each water change. After 28 days, all surviving snails were removed and used for shell size, shape, and crush resistance analyses.

Geometric Morphometrics

For field-collected snails from streams and wetlands in Oklahoma, all cercaria-shedding snails (wetland N = 83; stream N = 102) and a similar number of uninfected reference snails (wetland N = 87; stream N = 101) of similar size (± 0.25 mm) were photographed by placing individual snails next to a ruler with the shell aperture facing up and the shell apex facing away from the observer under an Olympus SZ51 stereoscope with an attached Nikon S4 Coolpix digital camera. Similarly, all the surviving snails from the laboratory experiment (N = 335/400) were photographed. To test for shape differences using geometric morphometrics (Rohlf and Marcus, 1993; Zelditch et al., 2004), photographed shells were digitized with landmarks according to Gustafson et al. (2014). We digitized 22 landmarks (Fig. 6.1) including 11 landmarks and 11 semilandmarks on digital images of each specimen using tpsDig2 (Rohlf, 2013). Snails that lacked homologous landmarks (i.e., discrete and easily recognizable endpoints) or semilandmarks (i.e., not a discrete endpoint; treated here as equidistant points along curves or whorls) because of broken shells, such as a broken aperture and/or apex, were

removed from the analyses (see Zelditch et al., 2004 for landmark discussion). Additionally, snails that were misaligned during the photograph process were removed, resulting in 346 field collected snails (Infected/Wetland N = 76; Infected/Stream N = 96; Uninfected/Wetland N = 79; Uninfected/Stream N = 95) and 335 laboratory-raised snails (Exposed/Nonflow N = 72, Exposed/Flow N = 95; Unexposed/Nonflow N = 75; Unexposed/Flow N = 93). We used tpsRelw to perform generalized least-squares Procrustes superimposition (i.e., rotation, translation, and scaling to remove positional effects; Bookstein, 1991) to obtain relative warp (RW) axes (Rohlf, 2010).

We calculated a divergence vector (DV), according to Langerhans (2009), as a measure of directional differences in shape across all RWs. Briefly, we performed a principal components analysis of the sums of squares and cross-products matrix of the main effects to derive an eigenvector of divergence (Langerhans, 2009). The resultant DVs describe linear combinations of dependent variables exhibiting the greatest shape differences in Euclidean space and avoids distortion of shape space. Nested analyses of covariance (ANCOVAs) were used to test for significant shape divergence. Centroid size (i.e., the square-root of the sum of squared distances from the landmarks to the centroid of the landmarks) was used as the measure of snail size. To account for geometric morphometric allometry, centroid size was included as a covariate in all shape analyses. Additionally, an ANOVA of centroid size was used to test for significant differences in shell size. For the field study, collection site was treated as a nested random effect within habitat type (i.e., wetland or stream). For the laboratory experiment, tank was treated as a nested random effect within the interaction of flow and trematode exposures. We used tpsRegr to visualize shape differences relative to DV scores (Rohlf, 2011).

Shell Crush-Resistance Assays

Each photographed snail was immediately evaluated using a crush-resistance assay. Only living snails with intact shells were used for analyses, resulting in 336 field-collected snails (Infected/Wetland N = 78; Infected/Stream N = 79; Uninfected/Wetland N = 83; Uninfected/Stream N = 96) and 306 laboratory-raised snails (Exposed/Nonflow N = 57, Exposed/Flow N = 95; Unexposed/Nonflow N = 63; Unexposed/Flow N = 91). To crush snails, we followed the methods described by Osenberg and Mittelbach (1989). Briefly, we placed each snail aperture down on a flat glass beaker and slowly poured sand into a flat glass beaker placed on top of the snail. Once the shell cracked, we weighed the mass of the sand and beaker and multiplied that value by the gravitational acceleration constant (9.8 m/s^2). We then calculated the crush force in Newtons required to crush each snail shell (Osenberg and Mittelbach, 1989). After snails were crushed, the soft tissues were examined under a stereo microscope for sporocysts, rediae, and cercariae to verify infection status. Nested ANCOVAs were used to test for crush resistance differences as previously described. All analyses were conducted in program R unless otherwise noted (R Developmental Core Team, 2015). Means and standard errors (SE) are presented throughout. All aspects of the research complied with protocols approved by the appropriate institutional animal care committee and adhered to the legal requirements of the country in which the research was conducted. No institutional, state, or national permits were required for this research.

RESULTS

Trematode Community Structure

Overall, we observed 7 cercaria types infecting *P. acuta* snails, with an overall prevalence of 4.4% (Table 6.2). All infected snails were infected with a single type of cercaria and no multiple infections were detected. 4.3% of wetland snails were infected with 1 of 6 cercaria types with prevalences ranging from 0-2.4%; 4.5% of stream snails were infected with 1 of 7 cercaria types with prevalences ranging from 0.1-2.2% (Table 6.2). Habitat accounted for ~17% of trematode community variation, whereas the first unconstrained axis accounted for ~24% (Fig. 6.2). Although *P. acuta* snails from wetlands and streams shared all but one of the trematode types (Table 6.2), their relative combinations and frequencies differed between the two habitats, resulting in significant differences in community structure (Pseudo $F_{1,22} = 4.1$, $P = 0.004$). In general, brevifurcate-apharyngeate, ornate, and spirorchid/schistosome type cercariae were more common in streams whereas echinostome type cercariae were more common in wetlands. Amphistome, armatae, and strigeid type cercariae were common to both habitat types (Fig. 6.2). For infected field-collected snails, the effect of cercaria type did not significantly affect snail size ($F_{23,126} = 0.91$, $P = 0.58$), crush resistance ($F_{25,120} = 0.83$, $P = 0.70$), or shape ($F_{23,126} = 0.92$, $P = 0.57$) at any site, and was removed from further analyses.

Laboratory Snail Survival

The percent of laboratory snails surviving 28 days in each tank was significantly affected by flow treatment ($F_{1,12} = 25.99$, $P < 0.001$), but not exposure to trematodes ($F_{1,12} = 0.004$, $P = 0.95$) or their interaction ($F_{1,12} = 0.19$, $P = 0.67$). Percent snail survival

within treatment was significantly higher in flow tanks (mean: 94% ± 1; range: 88-96%) than nonflow tanks (73% ± 4; 52-88%). For trematode-exposed laboratory snails, trematode infection did not significantly affect snails size ($F_{1,152} = 0.006$, $P = 0.94$), crush resistance ($F_{1,137} = 0.70$, $P = 0.40$), or shape ($F_{1,152} = 0.36$, $P = 0.55$) and therefore was removed from further analyses.

Shell Size

Because we systematically chose reference snails of similar size (± 0.25 mm) to match the size of infected snails for field-collected snails, infection status did not significantly affect snail size (Table 6.3). After accounting for the significant variation between sites, habitat was the only factor affecting snail size (Table 6.3). Wetland snails exhibited more variation in shell size than stream snails and, overall, wetland snails had significantly larger shells (16.0 mm ± 0.6) than stream snails (14.7 mm ± 0.3) (Fig. 6.3A). Laboratory snails exhibited relatively small shell sizes (13.2 mm ± 0.2) (Fig. 6.3B) and the only factor significantly affecting laboratory snail size was tank (Table 6.3).

Shell Crush Resistance

For field-collected snails, crush resistance was positively and significantly correlated with shell size (centroid size) ($R = 0.46$, $R^2 = 0.21$, $t_{312} = 9.19$, $P < 0.001$); thus, centroid size was included as a covariate in crush resistance analyses. The main effects of habitat, infection status, and their interaction significantly affected field-collected snail crush resistance (Fig. 6.3C, Table 6.3). Regardless of infection status, wetland snails had the most crush resistant shells (7.2 N ± 0.4; Fig. 6.3C). Under all conditions, stream snails had weaker shells than wetland snails. The crush resistance of uninfected stream snails was 80.9% the strength of wetland snails (5.8 N ± 0.3); whereas

infected stream snails had the weakest shells (4.3 N ± 0.4), reduced to 59.7% relative to wetland snails (Fig. 6.3C). Crush resistance also significantly increased with the date of capture (Table 6.3); however, the relationship was weak ($R = 0.17$; $R^2 = 0.03$). Snail shell size did not significantly increase with date of capture ($F_{1,344} = 0.33$, $P = 0.56$).

For laboratory snails, crush resistance was positively and significantly correlated with shell size (centroid size) ($R = 0.62$, $R^2 = 0.39$, $t_{305} = 13.88$, $P < 0.001$); thus, centroid size was included as a covariate in crush resistance analyses. Laboratory nonflow snails had significantly stronger shells than flow snails (Fig. 6.3D); however, the interaction of flow and trematode exposure was not significant (Table 6.3). Instead, trematode-exposed snails had modestly, but significantly, weaker shells (2.3 N ± 0.1) than non-exposed snails (2.4 N ± 0.1), regardless of flow (Fig. 6.3D, Table 6.3).

Shell Shape

The only significant factor affecting the shell shape of field-collected snails was habitat (Table 6.3, Fig. 6.3E). Visualization of significant shell shape differences based on habitat shows that stream snails exhibit wide apertures, wide bodies, short spires, and wide spire angles (Fig 4A). In contrast, wetland snails exhibit relatively more narrow bodies with tall spires and more acute spire angles (Fig. 6.4A).

After accounting for the significant covariation of shell size with shell shape, flow was the only significant factor affecting shell shape in laboratory-reared snails (Table 6.3). Visualization of significant shell shape differences based on flow showed that flow treatment snails exhibit wide apertures, wide bodies, short spires, and wide spire angles (Fig. 6.4B). In contrast, nonflow treatment snails exhibit relatively more narrow bodies with tall spires and more acute spire angles (Fig. 6.4B).

DISCUSSION

We tested if trematode parasitism in snails would exhibit environment-dependent effects, environment-independent effects, or not exhibit effects on functional shell characteristics of *P. acuta* snails. Parasitism did not induce any changes to shell size or shape, and only modestly reduced crush resistance (habitat-dependent in field snails; environment-independent in laboratory snails); however, flow environment exhibited significant effects on shell size, crush resistance, and shape of both wild and laboratory-raised snails.

Our field data indicate that snails from wetlands had large, crush resistant shells with narrow apertures and tall spires. In contrast, snails from streams had small, weak shells with wide apertures and short spires. Trematode parasitism had no apparent effect on the crush resistance of wetland-dwelling snails but significantly reduced the crush resistance of snails from streams. Additionally, parasitism had no significant effect on overall shell shape in stream- or wetland-dwelling snails. Laboratory *P. acuta* snails from nonflow tanks were also generally, but not significantly, larger than flow tank snails. Similar to our field study results, nonflow snails had significantly more crush resistant shells than flow snails. Additionally, the shapes of flow and nonflow snails significantly differed where nonflow snails exhibited shapes consistent with wetland snails and flow snails exhibited shapes similar to stream snails. For laboratory snails, trematode parasitism significantly reduced shell crush resistance regardless of their flow/nonflow environment.

It has been suggested that snail shell size plays an important role in reducing predation and maximizing fecundity (Alexander and Covich, 1991; DeWitt, 1991; Tripet

and Perrin, 1994). Previous research has demonstrated dramatic intraspecific differences in shell size based on habitat, particularly with regards to flowing and nonflowing environments, where gastropods from flowing habitats have relatively smaller shells (Dillon, 2011; Gustafson et al., 2014; Perez and Minton, 2008). Our field data further support these previous observations. In the laboratory, however, snails from flow treatments, although generally smaller, did not exhibit significantly smaller shells than snails from nonflow treatments. This could suggest that other environmental factors, in addition to, or correlated with, water flow contribute to size variation in nature (DeWitt, 1955). However, it could also be the case that we did not allow enough time for *P. acuta* snails to reach their maximum size. Additionally, laboratory *P. acuta* snails are known to exhibit less variation in shell morphology relative to wild snails (Clampitt, 1970; Gustafson et al., 2014); thus, limited variation may have led to non-significant differences. *Physa acuta* snails have also been shown to decrease in size when maintained under laboratory conditions (Clampitt, 1970; Gustafson et al., 2014). The cause of this reduction in size is currently unknown but could be related to differences in nutrition, space, predators, and water chemistry.

One of the most frequently reported effects of parasitism on snail shell morphology is an increase in size as a result of castration, referred to as gigantism (Minchella, 1985; Sorensen and Minchella, 2001). We were unable to test for the biological effects of trematode parasitism on the shell size of wild snails because we sampled parasitized and non-parasitized snails of similar size. In all cases, we were able to find uninfected snails of similar size to infected snails which might suggest gigantism is not occurring in these populations of *P. acuta* snails. However, the relative ages of

infected and non-infected field-collected snails were unknown. A previous laboratory experiment on *P. acuta* exposed to *H. eccentricus* showed a weak negative relationship between shell size and trematode infections also suggesting gigantism is not occurring in *P. acuta* snails (Gustafson and Bolek, 2015). Other pulmonate snails in the families Lymnaeidae and Planorbidae have been shown to exhibit gigantism (Sorensen and Minchella, 2001). However, to our knowledge, no study has observed trematode-induced gigantism in Physidae snails. In our laboratory study, *H. eccentricus* infected snails did not exhibit significant size differences compared to non-exposed snails, further supporting a lack of gigantism.

Shell crush resistance plays an important role in the defense of gastropods against several types of predators (Osenberg and Mittelbach, 1989; Rundle and Brönmark, 2001); however, crush resistance is infrequently investigated in shell plasticity studies. Our data suggest shell crush resistance is a highly plastic trait and that snails from nonflow environments (i.e., wetlands and nonflow tanks) exhibit stronger shells than snails from flow environments (i.e., streams and flowtanks) even after accounting for the covariation of shell crush resistance with shell size. We also found a relationship where the crush resistance of field-collected snails increased with date of capture. This could be a result of increased food availability, increased snail age, or calcium uptake throughout the season. This does not appear to be related to shell size because we did not detect an increase in shell size throughout the season. For field-collected snails, trematode parasitism had an interactive effect with habitat, significantly reducing the shell crush resistance of stream-dwelling snails, but not the shells of snails from wetlands. Unlike field-collected snails, there was no significant interaction between flow environment and

trematode exposure on the crush resistance of laboratory snails. However, there was a significant effect of trematode exposure on crush resistance. This could be a result of choosing a rediae-producing parasite (*H. eccentricus*) for laboratory experiments in contrast to a combination of sporocyst- and redia-producing trematodes in field studies. For example, physical castrators (i.e., rediae) could exhibit unique effects on host shell morphology relative to chemical castrators (i.e., sporocysts); however, this is currently unknown in *P. acuta* snails. Additionally, there could be different host-parasite interactions between *P. acuta* populations, or additional and unaccounted for complexity in stream habitats related to shell crush resistance. Trematode communities were significantly different between wetlands and streams and although parasite species specific effects could play a role (particularly between sporocyst- and redia-producing trematodes), we did not detect any significant differences in size, crush resistance, or shape of snails infected with different trematode cercaria types. Our sample size of specific cercaria types may have been too small for an adequate assessment. For example, only 22.7% of infected stream snails were infected with one of the three trematode cercaria types (i.e., brevifurcate-apharyngeate, ornate, spirorchid) primarily found in streams. Similarly, 24.7% of infected wetland snails were infected with echinostomes, which were the only trematodes highly associated with wetlands. Although the mechanism for decreased crush resistance of stream snails is currently unknown, it is likely related to differences in nutrition, foraging, water chemistry, or could be a result of the added energy investment of maintaining contact with habitat to avoid dislodgement (Brown and Quinn, 1988; Trussell, 1997). Alternatively, it is well known that shells in

flowing environments experience more wear on their shells and parasitism could have imposed an additional cost to shell maintenance.

Different aspects of snail shell shape have been shown to respond to abiotic and biotic factors. Predation and water flow are two of the most commonly studied factors affecting shell shape. In general, predator cues induce snails to form large bodies with narrow apertures, which is hypothesized to reduce reach-in predation (i.e., snails being pulled out of their shells, Langerhans and DeWitt, 2002). In contrast, water forces tend to induce snails to form small, wide bodied shells with wide apertures and short spires (Gustafson et al., 2014). Our field results confirm that habitat is a major driver of shell shape and supports our previous work on *P. acuta* wetland and stream ecotypes. Our previous common garden experiments demonstrate that offspring from stream-dwelling *P. acuta* snails lose their stream shell characteristics and converge with wetland ecotypes after a single generation in a nonflow laboratory environment, suggesting stream flow is the proximate cause of shell shape but a predator-defended phenotype (i.e., large, crush-resistant, and narrow) is the default phenotype (Gustafson et al., 2014). However, we did not raise the same offspring in a flowing environment and thus it is unclear if snails would have retained their stream-morph shape. In the current study, field-collected *P. acuta* snails were taken from a non-flowing wetland and their offspring experienced flow or nonflow laboratory treatments. After a single generation, we observed significant differences in shell shape between the two treatments. As expected, the shell shapes of laboratory snails in flowtank treatments exhibited wide bodies, wide apertures, and short spires, resembling the shape of wild stream snails. These results suggest wetland ecotype shell characteristics can also be lost in a single generation and shift to stream phenotypes.

We did not observe significant effects of parasitism on shell shape in wild or laboratory snails. This suggests that *H. eccentricus* and the trematodes detected in the field are not directly affecting the shell shapes of *P. acuta* snails. This is in contrast to previous studies which have all observed significant effects of trematode parasitism on some aspect of snail shell shape. For example, trematode parasitism (*Proterometra macrostoma*) in the freshwater snail *Elimia livesens* (= *Pleurocera semicarinate*; Dillon et al., 2013) has been shown to be correlated with increased aperture length (Krist, 2000). However, Krist (2000) field results did not correspond with her laboratory infections using the same species. Thus, it is difficult to determine if *P. semicarinate* snails with long apertures are more likely to become infected with *P. macrostoma* or if the infection was the cause of the morphological change. Although there is no direct study on the life history of *P. semicarinate,* it is currently suggested that *P. semicarinate* snails can live for up to 5 years in nature (Benson, 2015; Dazo, 1965). Other studies have focused on marine snails (e.g., Hay et al., 2005; Lagrue et al., 2007) which are also long-lived compared to *P. acuta* snails which live for a few months and exhibit multiple generations per growing season (Brown et al., 1988; Clampitt, 1970). Thus, it is possible that trematode induced shape effects take a longer time to materialize in infected snails. Our measure of shell shape is also a culmination of full shape space and it is possible we did not measure a specific morphometric that could have been affected.

Shell plasticity is not only important for gastropod defense, but it also has important taxonomic implications. Physid snails exhibit a worldwide distribution and it is well established that habitat has a dramatic impact on shell plasticity (Strong et al., 2008). For these reasons, snails in the family physidae are notoriously difficult to identify and it

is becoming increasingly evident that shell plasticity has led to historically unclear species identifications (Wethington and Lydeard, 2007). The difficulty at identifying physid species was acknowledged early in the Twentieth century when Crandall (1901) stated, "Species are supposed to be founded on permanent characteristics, [however,] every change of environment changes the *Physa*, and so long as environments continue to change, new forms will continue to occur." Although our results suggest that infection with trematodes is likely not a cause of *Physa* species misidentifications, future studies should infect uninfected wild snails from still and flowing water to confirm these results. It is clear, however, habitat, including water flow, must be taken into consideration when identifying *Physa* species.

In conclusion, we set out to assess the direct and interactive effects of trematode parasitism and flow and nonflow environment on functional shell characteristics of *P. acuta* snails, including shell size, crush resistance, and shape. For wild snails, parasitism only affected the crush resistance of stream snails. Similarly, parasitism only affected the crush resistance of laboratory snails. However, in contrast to infected field-collected snails which only had reduced crush resistance in streams, infected laboratory snails exhibited significantly weaker shells regardless of flow treatment. Finally, flow environment had significant effects on every shell characteristic we measured indicating habitat is the primary driver of *Physa acuta* shell morphology.

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Table 6.1. Locations, dates, and sample sizes of when and where *Physa acuta* snails were collected during the summer of 2014.

Site	Latitude (ºN)	Longitude (ºW)	Collection Dates (N)	Total N
Wetland				(1915)
A	36º06'25.27"	97º05'23.76"	17-Jun (85), 24-Jun (84), 7-Jul (263)	432
B	36º07'15.70"	97º01'29.19"	17-Jun (158), 24-Jun (170), 7-Jul (432)	760
C	36º06'10.12"	97º02'27.72"	18-Jun (194), 1-Jul (132), 7-Jul (78)	404
D	36º07'26.00"	97º03'18.56"	18-Jun (156), 1-Jul (163)	319
Stream				(2268)
A	36º06'22.41"	97º07'16.49"	17-Jun (60), 24-Jun (222), 7-Jul (216)	498
B	36º06'01.42"	97º04'47.38"	17-Jun (142), 24-Jun (186), 7-Jul (189)	517
C	36º07'16.54"	97º02'03.15"	18-Jun (279), 1-Jul (239), 7-Jul (191)	709
D	36º09'24.56"	97º04'40.79"	18-Jun (250), 1-Jul (138), 7-Jul (156)	544

Table 6.2. Prevalence and percent of community (percent of community in parentheses) of each cercaria morphotype in *Physa acuta* snails from wetlands or streams. Total prevalence of all cercaria types is summarized in the last column. The last row summarizes the prevalence of each cercariae type regardless of site or habitat.

Site	Armatae	Brevifurcate	Echinostomatidae	Ornate	Paramphistome	Spirorchid	Strigeid	All snails
Wetlands	2.40 (56.79)	0.00 (0.00)	1.05 (24.69)	0.05 (1.23)	0.16 (3.70)	0.05 (1.23)	0.52 (12.35)	4.34
A	6.51 (100)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	6.51
B	1.05 (34.78)	0.00 (0.00)	1.32 (43.48)	0.13 (4.35)	0.26 (8.69)	0.00 (0.00)	0.26 (8.69)	3.29
C	0.99 (25.00)	0.00 (0.00)	1.98 (50.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.99 (25.00)	3.96
D	1.88 (42.86)	0.00 (0.00)	0.63 (14.29)	0.00 (0.00)	0.31 (7.14)	0.31 (7.14)	1.25 (28.57)	4.39
Streams	2.20 (56.82)	0.13 (3.41)	0.04 (1.14)	0.40 (10.23)	0.31 (7.95)	0.35 (9.09)	0.44 (11.36)	4.50
A	3.01 (65.22)	0.00 (0.00)	0.20 (4.35)	0.20 (4.38)	0.00 (0.00)	0.00 (0.00)	1.2 (26.09)	5.02
B	2.51 (50.00)	0.38 (7.69)	0.00 (0.00)	0.58 (11.54)	0.77 (15.38)	0.19 (3.85)	0.58 (11.54)	5.22
C	1.97 (51.85)	0.00 (0.00)	0.00 (0.00)	0.71 (18.52)	0.28 (7.41)	0.71 (18.52)	0.14 (3.70)	5.22
D	1.47 (66.67)	0.18 (8.33)	0.00 (0.00)	0.00 (0.00)	0.18 (8.33)	0.37 (16.67)	0.00 (0.00)	2.39
All snails	2.23 (56.80)	0.07 (1.78)	0.50 (12.43)	0.24 (5.92)	0.24 (5.92)	0.22 (5.33)	0.48 (11.83)	4.42

Table 6.3. Summary of nested ANCOVAs testing the effects of flow environment and trematodes on snail shell size, crush resistance, and shape. Centroid size and date are treated as covariates; (site) is nested within habitat and (tank) is nested within the interaction of the main effects. Degrees of freedom (treatment, error) are reported with F statistic. Centroid size was not included as a predictor in the size analysis (--).

Variables	Shell size Centroid size		Shell crush resistance Newtons		Shell shape DV	
	F	*P*	*F*	*P*	*F*	*P*
Field study						
Habitat	1,335 = 11.4	<0.001	1,300 = 86.68	<0.001	1,335 = 36.30	<0.001
Infected	1,335 = 0.03	0.86	1,300 = 9.09	0.003	1,335 = 3.22	0.07
Hab x inf	1,335 = 0.05	0.82	1,300 = 6.23	0.01	1,335 = 0.85	0.34
Centroid size	--	--	1,300 = 117.49	<0.001	1,335 = 0.38	0.52
Date	1,335 = 0.04	0.85	1,300 = 4.39	0.04	1,334 = 5.42	0.02
(Site)	6,335 = 35.42	<0.001	6,300 = 32.54	<0.001	6,335 = 1.58	0.2
Laboratory experiment						
Flow	1,319 = 0.67	0.41	1,288 = 40.46	<0.001	1,318 = 17.84	<0.001
Exposed	1,319 = 1.24	0.27	1,288 = 5.57	0.02	1,318 = 1.19	0.27
Flow x exp	1,319 = 0.13	0.71	1,288 = 0.33	0.56	1,318 = 0.73	0.39
Centroid size	--	--	1,288 = 211.29	<0.001	1,318 = 30.87	<0.001
(Tank)	12,319 = 2.56	0.003	12,288 = 3.69	<0.001	12,318 = 1.25	0.25

Fig. 6.1. Landmark locations for wild and laboratory *Physa acuta* snails on a typical photograph. Homologous landmarks (N = 11) are represented by circles; semilandmarks (N = 11) are represented by squares. Scale in mm.

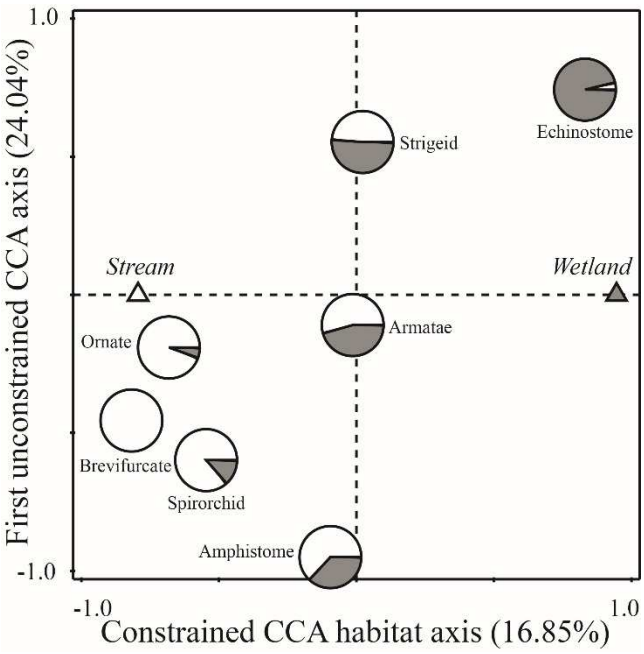


Fig. 6.2. Partial canonical correspondence analysis pieplot showing the significant differences in trematode community structure between all infected *Physa acuta* snails collected from streams and wetlands. Pies represent the relative frequency of each cercaria type in either streams (white) or wetlands (gray). Percent of the explained variation is indicated in parentheses on each axis.

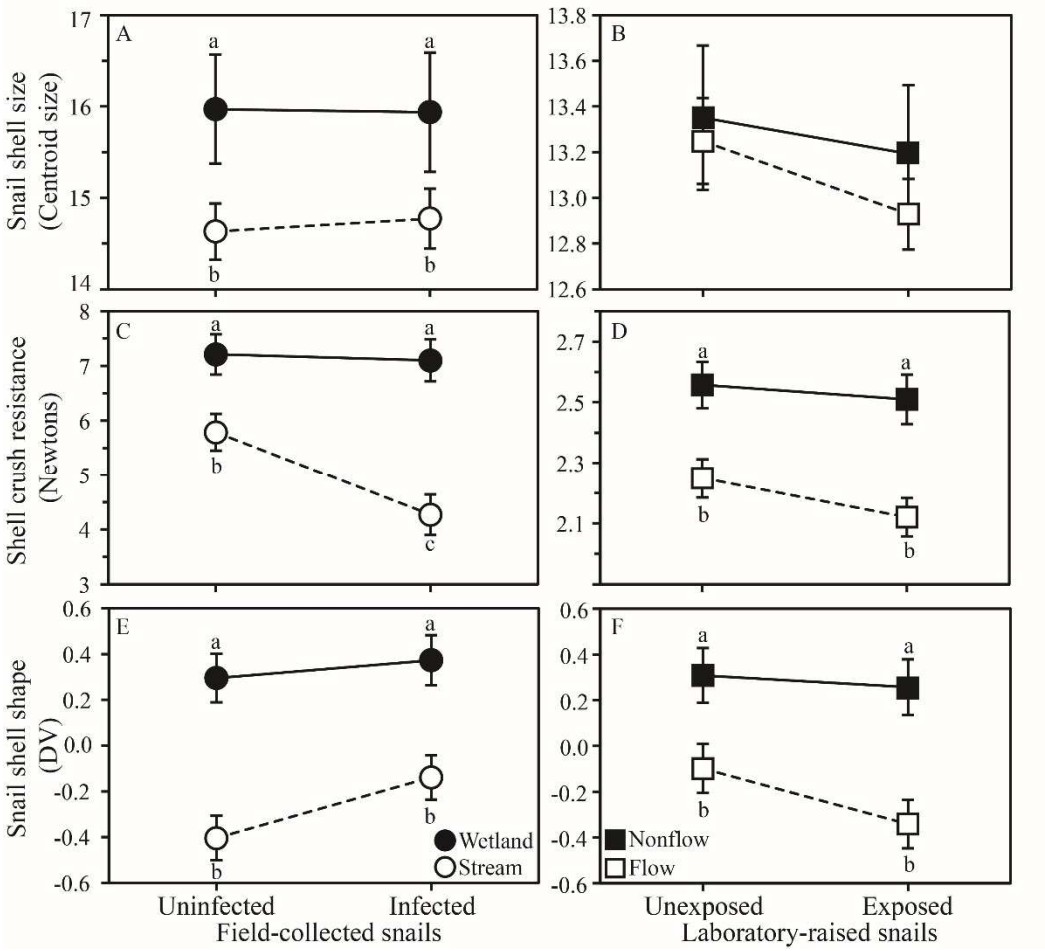


Fig. 6.3. Effects of trematode infection status and flow or non-flow environment on snail (A, B) shell size, (C, D) shell crush resistance, and (E, F) shell shape. Field-collected snails were either from wetlands (filled circle) or streams (open circle) and either uninfected or infected. Laboratory-raised snails were from nonflow (filled square) or flow (open square) tanks and were either not exposed or exposed to trematodes. Lowercase letters indicate significantly homogenous groups based on Tukey's HSD post hoc tests. Means ± SEs are presented.

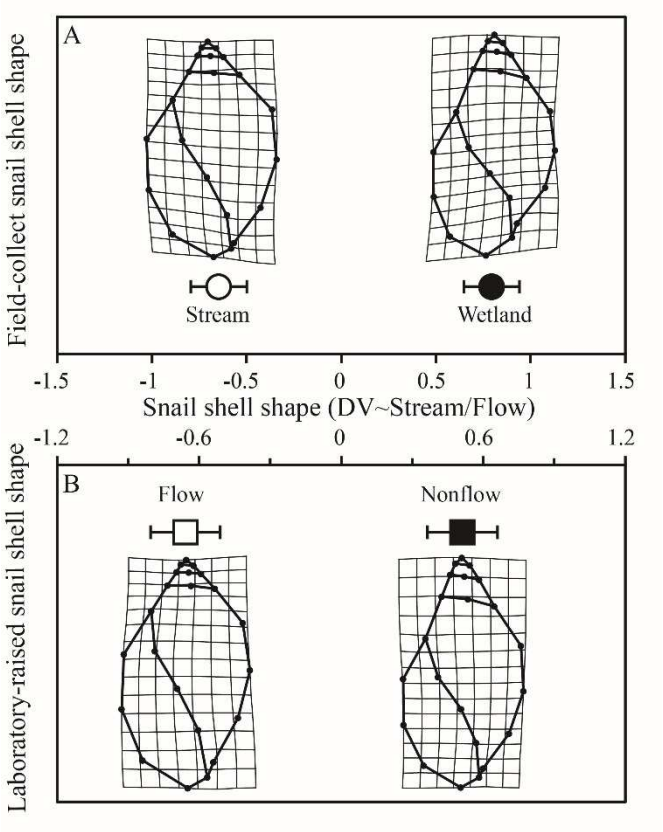


Fig. 6.4. Significant shell shape differences of *Physa acuta* snails from flow or nonflow environments. (A) Field-collected snails are from streams (open circle) or wetlands (closed circle); (B) laboratory-raised snails are from flow (open squares) or nonflow (closed squares) tanks. Thin plate spline deformation grids are the representative mean shapes for each group. Divergence vectors (DVs) were calculated separately for field and laboratory snails and are based on shape differences between habitats (streams/wetlands) or flow tanks (flow/nonflow). Means ± SEs are presented.

CHAPTER VII

CONCLUSIONS

EFFECTS OF ATRAZINE ON HOSTS, PARASITES, AND HOST–PARASITE INTERACTIONS

My work investigated how environmental factors, including natural and anthropogenic, affect host–parasite interactions. Throughout my research, I primarily used snails and trematodes as a model system. The aims of my first three chapters were to understand how atrazine, a commonly used herbicide, affected (1) snails and ostracod potential hosts, (2) their parasites, (3) parasite transmission, and (4) host–parasite interactions (including interactive effects of atrazine and parasitism on hosts, the production of parasites from hosts, and parasite infectivity to hosts).

First, in CHAPTER II, I explored the effects of atrazine on hosts (snails) and presented a review on the effects of atrazine on freshwater snails. Studies indicate that atrazine affects snails from the subcellular to the community level and atrazine-snail responses appear to be similar among major freshwater snail families. At lower biological levels, environmentally relevant atrazine concentrations can affect enzyme activity, immunity, can cause cell and tissue damage, and can modify snail behavior in the absence of other stressors. However, many studies, including mine, suggest suborganismal effects rarely translate into individual or population level effects such as growth, reproduction, or survival. This is particularly true for environmentally relevant

atrazine concentrations which did not elicit life history effects on a broad range of species in the absence of other stressors. However, it is important to keep in mind that the effects on natural populations may not occur directly through atrazine as an active chemical, but through other compounds that are inherently associated with their presence in the field (e.g., solvents, surfactant), or with additional stressors. Interestingly, some studies suggest that atrazine effects reappear at the aquatic community level and indirectly or interactively affect snail reproduction, survival, and biomass at environmentally relevant concentrations. Given the importance of snails in nutrient cycling, food webs, and parasite transmission, indirect effects of atrazine, and other pesticides, have the broadest implications for downstream or cascading effects on aquatic ecosystem dynamics and parasite transmission.

The next step in evaluating the effects of atrazine on host–parasite interactions was to address the reality that snails experience variation to trematode exposures in nature. The goal of CHAPTER III was designed to better understand how to quantitatively expose snails to different trematode egg doses resulting in snail cercariae production without "over-infecting" and killing the snail host. My laboratory infections suggest there is a distinct tradeoff where higher doses of trematode eggs may be more likely to result in a patent infection, but also increases the odds of killing the host before releasing cercariae or early on in the infection. On the other hand, low egg dose exposures may not reduce snail host fitness, but may not be enough to establish an infection. Although we report fewer shedding days for snails exposed to higher trematode egg doses, there may be potential differences in the number of cercariae released per day, which we did not record. For example, although a heavily infected snail

might die within a day or so, it could shed more cercariae than a lightly infected snail that releases only a few cercariae over a longer period of time. This would be an interesting avenue for further study. Overall, this study gives insight into the potential *H. eccentricus* egg doses snails experience in nature and has important implications for future laboratory studies using non-domesticated trematode life cycles. This work indicates that trematode dose must be accounted for when studying the effects of extrinsic factors on host–parasite interactions.

After establishing hypotheses and expectations based on snail toxicity tests and quantitative infection assays, I examined the effects of atrazine on host–parasite interactions. Previous research on the effects of chemicals on hosts, parasites, or parasite transmission has either exposed the host or the parasite, not both. However, in nature, both the host and parasite could experience similar concurrent chemical concentrations. Thus, I tested the effects of atrazine (0, 3, 30 μg/L; ecologically-relevant concentrations) on multiple aspects of a trematode (*Halipegus eccentricus*) life cycle. My study demonstrated that each trematode life stage and each host species responded differently to atrazine exposures. Additionally, throughout a trematode life cycle, each life stage typically infects a different host and those unique host–parasite relationships can be affected by pesticides. In this study, there were multiple indications of reduced transmission, including reduced production of cercariae and reduced metacercarial infectivity. However, there was no indication of enhanced transmission. Thus, for *H. eccentricus* (and potentially other species), atrazine (and potentially other pesticides) appears to reduce trematode transmission which has important implications for the maintenance of trematode biodiversity in altered ecosystems. This study shows that

environmental effects on host–parasite interactions should be examined at each stage and provides a testable mechanism to better understand altered trematode transmission in contaminated ecosystems.

EFFECTS OF HABITAT ON PARASITES, HOSTS, AND HOST–PARASITE INTERACTIONS

The second part of my dissertation focused on natural environmental effects on hosts, parasites, and host–parasite interactions. The aims of the last two chapters of my dissertation were to understand the effects of habitat on host morphology, parasite distributions, and parasite-induced morphologies.

It is well established that animals require certain habitats to survive and reproduce. Some animals require specific habitats and some can thrive in many different habitats. However, it is not always clear if species are segregated based on their ecological niches or if the individuals from a single species are developing different phenotypes under different environmental conditions. For snails, aspects of shell morphology may be plastic or genetically fixed. Even within a single population, environmentally-induced shell shape plasticity can lead to unclear species identifications as a result of extreme shape variation. Based on observational differences in shell size and shape between wetland and stream snails, I conducted a common garden experiment to test whether there were two distinct *Physa* species segregated based on their ecological niches or if the snails were from a single species and the environment was creating distinct ecomorphs. My results indicated that wetland and stream snails belong to a single species with a large amount of phenotypic plasticity in shell morphology. These snails converged with the shape of wetland snails after only a single generation. Although snails

from both populations became smaller, there was a single generation lag in size convergence, suggesting either a maternal or other epigenetic difference between generations, but not a classical genetic difference between populations. Although I cannot rule out that other environmental factors were involved in the shell shapes expressed in our laboratory-reared snails, our results suggest that water current (or lack of current and/or associated correlates) has a large impact on snail shell morphology.

After evaluating environmental effects on snail shell morphology, I investigated the interactive effects of environment and parasitism on snail shell morphology. If trematode parasitism has a unidirectional effect on shell size, strength, or shape, then the host could be maladapted to certain environments resulting in reduced trematode transmission. In contrast, trematodes could cause snails to express unique phenotypes based on their environments, which could increase their probability of transmission. In CHAPTER VI, I conducted a field study and complementary laboratory experiment to address the effects of trematode parasitism on functional shell characteristics (size, crush resistance, and shape) of *Physa acuta* snails in flow and non-flow environments. For wild snails, parasitism only affected the crush resistance of stream snails. Similarly, parasitism only affected the crush resistance of laboratory snails. However, in contrast to infected field-collected snails which only had reduced crush resistance in streams, infected laboratory snails exhibited significantly weaker shells regardless of flow treatment. Finally, flow environment had significant effects on every shell characteristic I measured indicating habitat is the primary driver of *P. acuta* shell morphology.

CLOSING REMARKS

Overall, the first three chapters of my dissertation broaden our understanding of toxicological effects on snails, trematode life cycles, and host–parasite interactions. Our paper in *Ecotoxicology* (CHAPTER II) evaluated both acute and chronic atrazine toxicity assays to snails in the same study. Additionally, we evaluated all the snail literature in a way to better understand the effects of ecologically-relevant atrazine concentrations on snails at multiple levels of biological organization. Our study published in the *Journal of Parasitology* (CHAPTER III) established a dose–response relationship between trematode dose and host–parasite interactions, and specifically for *Halipegus eccentricus*, a trematode species which is becoming a model system for host–parasite interaction studies. Additionally, our paper in *Parasitology Research* (CHAPTER IV) was the first paper to simultaneously test the effects of a chemical on hosts and parasites and on multiple host species and trematode stages. Finally, the last two chapter of my dissertation broaden our understanding of natural environmental effects on hosts and host–parasite interactions. Our work published in *Evolutionary Ecology Research* (CHAPTER V) demonstrated that the shell shapes of offspring from distinct snail morphotypes converge within a single generation. Lastly, our paper in the *Journal of Morphology* (CHAPTER VI) was the first field- and laboratory- assessment of environmental and parasitological effects on multiple snail shell characteristics. As a result, this dissertation provides contributions to the fields of ecology, parasitology, toxicology, and morphometrics.

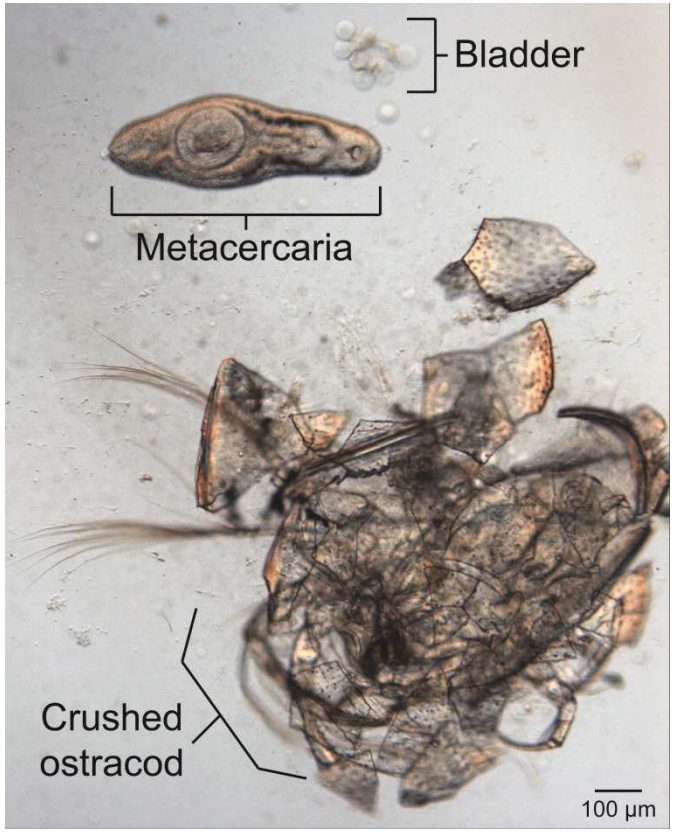
APPENDICES

A2.1. Mean summaries of measured atrazine concentrations, water temperature (T_w), dissolved oxygen (mg/L), and ammonia (NH_3-N) at each nominal concentration for each species. Water temperature and dissolved oxygen were randomly sampled twice from 4 bowls at each concentration for each species. Atrazine and ammonia were randomly sampled once from 3 bowls at each concentration for each species. Atrazine concentrations were below quantitation limit (<0.05) and standard deviations could not be computed (--) for the control.

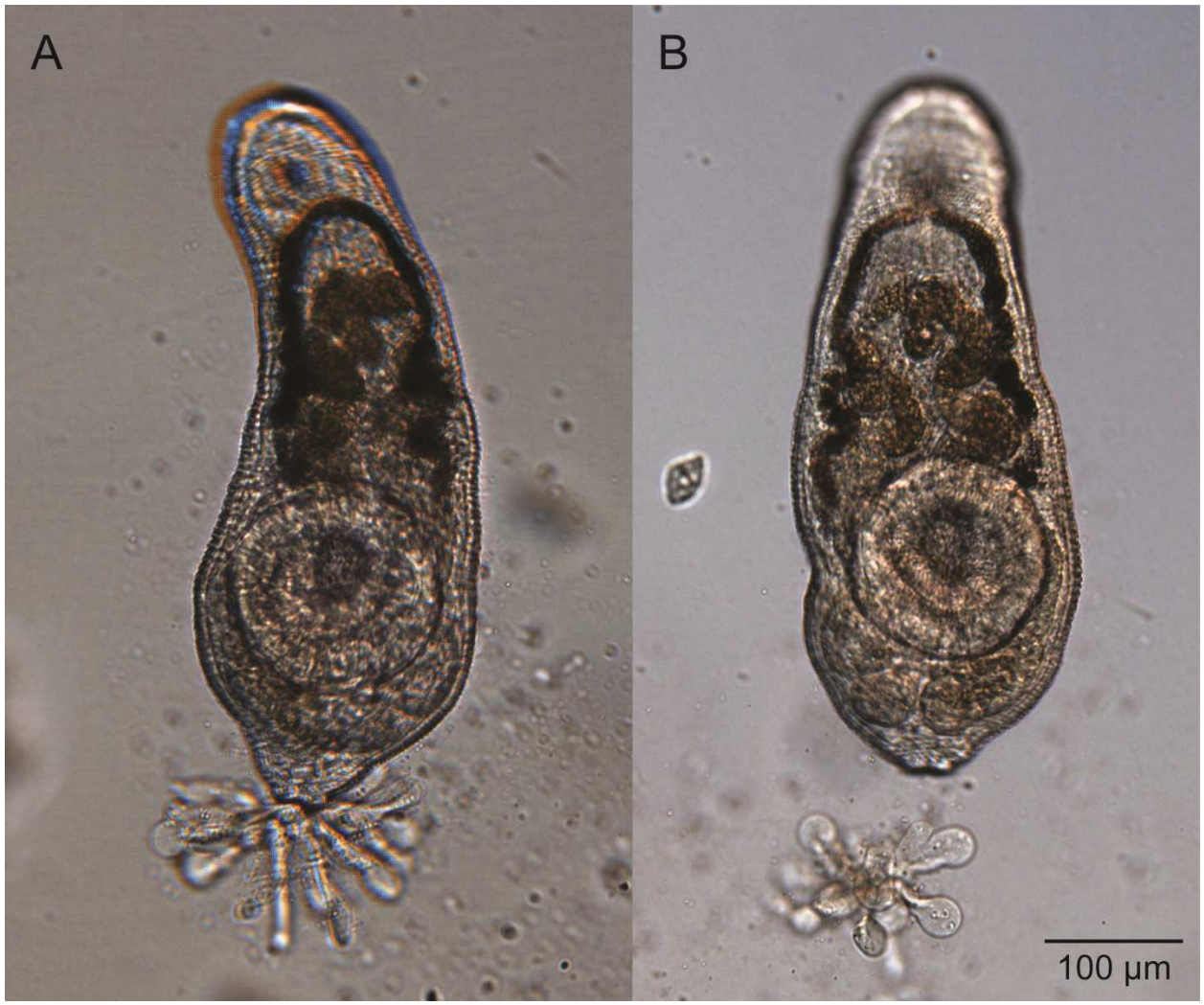
Species	Atrazine concentration (µg/L)			T_w	SD	DO	SD	NH_3-N	SD
	Nominal	Measured	SD						
Biomphalaria glabrata									
	0	<0.05	--	24.68	0.87	7.18	0.22	0.16	0.09
	0.3	0.31	0.00	24.85	0.81	7.07	0.27	0.11	0.07
	3	3.09	0.08	24.94	0.80	7.10	0.22	0.16	0.11
	30	29.93	1.38	25.08	0.95	7.09	0.31	0.16	0.12
Helisoma trivolvis									
	0	<0.05	--	25.60	0.41	7.16	0.29	0.17	0.06
	0.3	0.31	0.01	25.43	0.37	7.19	0.23	0.17	0.09
	3	3.22	0.12	25.45	0.30	7.12	0.16	0.14	0.07
	30	30.45	0.43	25.56	0.34	7.07	0.20	0.14	0.10
Physa acuta									
	0	<0.05	--	24.61	1.28	7.06	0.32	0.14	0.07
	0.3	0.31	0.01	25.08	0.37	7.14	0.34	0.15	0.08
	3	3.09	0.10	25.21	0.30	7.09	0.18	0.16	0.07
	30	31.24	0.29	25.24	0.59	7.10	0.24	0.14	0.09
Stagnicola elodes									
	0	<0.05	--	24.68	0.87	7.18	0.22	0.15	0.10
	0.3	0.30	0.01	24.85	0.81	7.07	0.27	0.14	0.06
	3	3.09	0.11	24.94	0.80	7.10	0.22	0.14	0.10
	30	29.72	1.22	25.08	0.95	7.09	0.31	0.15	0.07

A2.2. Mean summaries of survival, size, and reproductive parameters for control snails through time. Size was not measured during the first water change of the week and is therefore not reported (--).

Endpoint	Week 1				Week 2				Week 3				Week 4			
Species	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Survival (%)																
Biomphalaria glabrata	88.6	10.7	75.7	16.2	67.1	24.3	61.4	28.5	60.0	27.7	60.0	27.7	60.0	27.7	54.3	31.5
Helisoma trivolvis	100.0	0.0	97.5	4.6	97.5	4.6	96.3	7.4	82.5	34.9	81.3	34.4	80.0	35.1	77.5	33.7
Physa acuta	100.0	0.0	97.5	7.1	95.0	9.3	95.0	9.3	93.8	9.2	93.8	9.2	92.5	10.4	90.0	12.0
Stagnicola elodes	96.3	5.2	91.3	6.4	91.3	6.4	87.5	8.9	86.3	9.2	86.3	9.2	86.3	9.2	86.3	9.2
Size (mm)																
Biomphalaria glabrata	13.8[a]	0.4	13.7	0.5	--	--	14.1	0.8	--	--	14.0	0.8	--	--	14.7	0.7
Helisoma trivolvis	11.5[a]	0.2	11.8	0.3	--	--	12.0	0.2	--	--	12.1	0.3	--	--	12.1	0.6
Physa acuta	3.7[a]	0.2	4.6	0.6	--	--	5.6	0.5	--	--	6.2	0.4	--	--	6.3	0.4
Stagnicola elodes	5.2[a]	0.1	5.4	0.4	--	--	7.3	1.1	--	--	10.6	1.5	--	--	11.7	1.3
Cumulative egg masses per snail																
Biomphalaria glabrata	0.1	0.2	0.2	0.4	0.3	0.5	1.6	2.0	1.8	2.1	1.9	2.1	3.9	2.6	6.3	4.5
Helisoma trivolvis	3.9	1.4	6.3	2.7	6.7	2.7	7.1	3.0	8.7	3.6	9.5	3.4	10.7	4.0	10.8	4.1
Physa acuta	0.9	0.7	1.8	1.0	3.5	1.5	5.4	2.7	7.9	3.0	10.6	3.5	12.2	3.5	14.1	4.0
Stagnicola elodes	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Cumulative eggs per snail																
Biomphalaria glabrata	3.9	7.2	12.7	23.9	23.2	37.6	36.7	43.7	45.8	47.1	53.2	48.6	61.8	51.7	70.3	54.9
Helisoma trivolvis	18.6	4.7	36.7	5.1	47.1	11.2	57.4	13.7	69.9	16.1	80.6	16.7	86.0	22.8	98.1	28.6
Physa acuta	25.5	10.9	38.6	17.1	54.3	22.9	70.7	28.9	84.9	29.9	99.5	29.4	112.8	30.7	124.2	31.3
Stagnicola elodes	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	2.0	3.8	4.1	7.9	4.8	8.8	10.4	19.7



A4.1. Photograph of a recently crushed ostracod infected with a single *Halipegus eccentricus* metacercaria. The metacercaria pinched off its skirt-like bladder while crawling between the glass slide and glass cover slip.



A4.2. Photographs of *Halipegus eccentricus* metacercariae removed from ostracods. The metacercariae are either not infective (A) or infective (B) based on the condition (retained or removed; respectively) of the skirt-like bladder.

A4.3. Mean summaries of measured atrazine concentrations, water temperature (T_w), dissolved oxygen (mg/L), and ammonia (NH_3-N mg/L) at each nominal concentration. Atrazine was randomly sampled from 4 bowls at each concentration once immediately after a water change (t0) and once after the longest period between water changes (i.e., 4 days; t+4). Water temperature and dissolved oxygen were randomly sampled twice from 4 bowls at each concentration at t+4. Ammonia was randomly sampled once from 3 bowls at each concentration at t+4. Atrazine concentrations were below quantitation limit (<0.05 µg/L) and standard deviations could not be computed (--) for the control.

Atrazine concentration (µg/L)					T_w	SD	DO	SD	NH_3-N	SD
Nominal	Measured t0	SD	Measured t+4	SD						
0	<0.05	--	<0.05	--	25.13	0.41	7.05	0.33	0.17	0.08
3	2.90	0.17	2.89	0.04	25.18	0.37	7.11	0.34	0.18	0.08
30	28.6	0.36	27.4	0.65	25.22	0.30	7.06	0.25	0.16	0.09

VITA

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