Infection dynamics of a stream trematode, *Metagonimoides oregonensis,* and plethodontid salamander second intermediate hosts

Jennifer A. Wyderko

Thesis submitted to the faculty of the Virginia Polytechnic Institute and State University in partial fulfillment of the requirements for the degree of

Master of Science in Biological Sciences

Lisa Kay Belden, Chair
Ernest F. Benfield
Jeremy M. Wojdak

12 April 2013
Blacksburg, Virginia

Keywords: trematode, parasite, behavior, salamander, behavior

ABSTRACT

Infection dynamics of a stream trematode, *Metagonimoides oregonensis,* and plethodontid salamander second intermediate hosts

Jennifer A. Wyderko

Metagonimoides oregonensis is a digenetic trematode that infects raccoons as definitive hosts, the snail *Elimia proxima* as a first intermediate host and in the southern Appalachians, encysts in the muscle tissue of a variety of second intermediate salamander hosts. My first study examined 289 individual salamanders representing six species from 23 streams in North Carolina to determine which species of salamanders are naturally infected. I found that five of the six species examined had natural infections, and it is likely the sixth species is a potential host as well. I did see different patterns of infection among the species, but found that *Desmognathus quadramaculatus* may be most important in transmission, as they had the highest prevalence and intensity of infection. This may be due to their long larval period, which results in a longer trematode accrual period. My second study explored the role of host and parasite behavior in driving infection dynamics in this system. I examined both parasite response to host chemical cue and host response to parasite presence and chemical cue. I did not see a behavioral response by either the parasite or the host, indicating behavior is probably not an important factor in explaining infection distribution in this system, and that distributions are probably a result of other environmental or ecological factors. My third study examined the effect of cercariae exposure (n=0, 20, 60) on locomotor performance of *D. quadramaculatus, Eurycea wilderae* and *Hyla versicolor.* I did not see any effect on performance for any of the species.

ACKNOWLEDGMENTS

I would like to thank Lisa Belden for all of her support and guidance as both an undergraduate and graduate advisor. I am grateful to my committee members Jeremy Wojdak for his tireless assistance with R programming and Fred Benfield for his helpful comments on numerous drafts. I would like to thank all of the people who helped with animal collections: Whitney Beasley, Ivonne Cedillos, Letitia Clay, and Scooter Cogar. I would also like to thank Sally Zemmer for always being available to offer advice and support. I am grateful to my friends and family members for their support and patience. And to my dogs for letting me practice my defense talk with them numerous times.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ATTRIBUTION

Co-authors for chapter two, "Variable infection of stream salamanders in the southern Appalachians by the trematode *Metagonimoides oregonensis* (Family: Heterophyidae)" are E. F. Benfield, K. C. Cecala, J. C. Maerz, and L.K. Belden. Kristen Cecala and John Maerz is a professor at the Warnell School of Forestry at the University of Georgia and Kristen Cecala is a former PhD student. They collected the salamanders used in this study and provided valuable feedback on the manuscript.

Co-authors for chapter three, "Effects of *Metagonimoides oregonensis* exposure on amphibian locomotor performance" are I. Cedillos and L. K. Belden. Ivonne Cedillos is an undergraduate researcher in the lab of Dr. Lisa Belden and she assisted with tadpole activity performance tests.

Co-authors for chapter four, "Host-parasite behavioral interactions between *Metagonimoides oregonensis* cercariae and the second intermediate salamander host, *Desmognathus quadramaculatus*" are J.M. Wojdak and L.K. Belden.

1. INTRODUCTION

Parasites represent a surprisingly large portion of the diversity and biomass of natural systems (Kuris et. al. 2008). They fulfill diverse and varied ecological roles, and can have large effects on community structure and ecosystem function (Minchella and Scott 1991, Marcogliese 2004, Hudson et al. 2006). Almost all organisms are at risk of being parasitized at some point in their life, so the implications for the role of parasites in shaping ecological interactions is huge. In recent years parasites have begun to be integrated into our understandings of food web complexity, especially in the case of trophically transmitted parasites (Marcogliese and Cone 1997, Mouritsen and Poulin 2004, Lafferty 2008).

Parasitic infection tends to result in direct phenotypic modifications to the host that may alter behavior, morphology, or physiology (Thomas 1991). While these modifications often have direct impacts on the host, they can also result in cascading trait-mediated indirect effects that alter community structure or interactions. For example, infection of *Littorina littorea* snails by the trematode, *Cryptocotyle lingua*, results in degradation of their digestive tract causing a decrease in foraging. These snails graze primarily on one species of algae, so a decrease in foraging results in a cascade of compositional changes to the macroalgal community structure (Wood et al. 2007). These types of cascading changes on community structure also have implications for alterations to food web structure and strength. Parasites can also alter food web links by mediating predator-prey interactions. Parasitic modifications to the host often result in hosts that are more conspicuous or susceptible to predation. For trophically transmitted parasites, selection may favor host (prey) alterations that increase transmission to a subsequent host (predator).

Parasites with high host-specificity have experienced selection to ensure successful transmission to the proper host. This selection pressure is very strong because if a parasite does not find a suitable host it cannot reproduce. Parasites with complex life cycles are often transmitted through trophic interactions. For continued survival the parasite must maximize transmission by maximizing the odds that its host will be eaten (Lewis 2002). In many systems, this results in parasites that alter intermediate host behavior, making them more vulnerable to predation by the definitive host. Whether or not these behavioral modifications are adaptive is still an area of debate. Predators will consume the most profitable prey for them – usually the prey that costs them the least amount of energy (Hudson 1992). One of the best examples of host manipulation can be found in ants that are the second intermediate host for the liver fluke, *Dicrocoelium dendriticum.* The parasite causes infected ants to climb up and attach to a stalk of grass. This makes it more likely that a cow will consume the parasite as it is grazing (Spindler 1986).

Hosts can also display avoidance behavior towards parasites. These behaviors may include selective foraging, migration, avoidance of territories, and grooming (Daly 2010). For hosts infected by free-living parasites these behaviors may be even more obvious. For example, some breeding amphibians avoid areas with trematodes (Kiesecker and Skelly 2000). Negative pathology is often directly related to the number of parasites that succeed in infecting the host, so defensive behaviors probably play an important role in controlling infection (Daly 2010).

Trematodes are a class of parasitic flatworms with complex life cycles, usually involving at least three hosts, although the life cycle varies among species. *Metagonimoides oregonensis* is a trematode that uses raccoons as a definitive host, where sexual reproduction occurs in the small intestine. Raccoons shed the eggs of the adult worms in feces into the environment. Miracidia

hatch and infect the first intermediate host, *Elimia proxima,* a stream-dwelling snail. Miracidia migrate to the digestive glands of the snail and there develop into rediae. Cercariae develop in these rediae and hatch out through the birth pore. The cercariae can then either leave the snail and infect another host, or re-encsyt in the snail tissue as metacercariae (Burns and Pratt 1953). The use of a distinct second intermediate host seems to vary across the range of *Metagonimoides* (Burns and Pratt 1935, Lang and Gleason 1967). In North Carolina, plethodontid salamanders appear to be a common secondary host for the trematode (Goater et al. 1987, Belden et al. 2012). Once the cercariae find a salamander they encyst as metacercariae in the muscle tissue where they remain dormant until a raccoon consumes the salamander.

While the definitive and first intermediate hosts of trematodes are usually fairly specific, trematodes are often more general in their second intermediate host use. However, the distribution of the parasite among potential second intermediate hosts tends to be variable and there is usually one species that is a more common or preferred host (Evans and Gordon 1983). In order for a host to be utilized by a parasite, the parasite must first encounter this host and the host must be susceptible to infection. Reasons for variable distributions of infection among potential hosts are probably system specific and influenced by a number of abiotic and biotic factors. Temporal patterns, potential host abundance, behavior, and microhabitat use are just a few possible factors influencing parasite distribution among potential hosts (Detwiler and Minchella 2009).

Predator-prey interactions in aquatic systems have been studied at length (Wilbur 1972). Chemical cues play an important role in shaping predator-prey interactions in aquatic systems. For amphibians, chemical signals can provide information about both interspecific and intraspecific interactions. Many amphibian species can recognize chemical cues of injured

conspecifics and display avoidance behavior of that area (Lutterschmidt et al. 1994), and some species can recognize congeners in a nearby area (Marvin et al. 2004). Intraspecific communication also influences predator-prey interactions because many amphibians learn to detect chemical signals from frequent predators (Turner 2008, Mirza et al. 2006).

Similarly, many amphibians recognize the presence of parasites in their environment and display avoidance behaviors. There is some evidence that they detect a chemical stimulus, however, further research is needed (Rohr et al. 2008). *Hyla versicolor* can distinguish between trematode-infected and uninfected snails and will preferentially lay eggs away from cercariae shedding snails (Kiesecker and Skelly 2000). *Bufo americanus* tadpoles demonstrate avoidance behaviors in the presence of larval trematodes (Rohr et al. 2008). *Rana clamitans* and *Rana sylvatica* tadpoles become less active in an environment with cercariae, which may hinder detection by the parasites (Thiemann and Wassersug 2000). Once cercariae have made contact, tadpoles exhibit defensive swimming patterns to dislodge the parasites before penetration. (Taylor et al. 2004). This avoidance behavior can be costly for tadpoles because defensive swimming may attract predators. In the presence of predators, tadpoles cease defensive behaviors and so are more likely to become infected (Thiemann and Wassersug 2000). *Rana sylvatica* tadpoles experience significantly higher mortality rates in the combined presence of trematodes and predator than with either factor alone (Belden and Wojdak 2011, Raffel et.al. 2010). Population density can also play a role on the effects of parasitism on amphibians. *Rana pipiens* experience increased mortality from the trematode *Echinostoma trivolvis* when there is a high density of conspecifics nearby (Koprivnikar et al. 2008). These studies indicate that amphibians may undergo behavioral changes in response to the presence of trematodes.

The objectives of these studies was to (1) determine the distribution of *Metagonimoides oregonensis* among several potential salamander second intermediate hosts (Chapter 2), (2) examine the effect of metacercariae infection on salamander locomotor performance (Chapter 3), and (3) explore behavioral interactions of both the salamander host and parasite to determine the role of behavior in transmission (Chapter 4).

2. VARIABLE INFECTION OF STREAM SALAMANDERS IN THE SOUTHERN APPALACHIANS BY THE TREMATODE *METAGONIMOIDES OREGONENSIS* (FAMILY: HETEROPHYIDAE)

J. A. Wyderko, E. F. Benfield, K. C. Cecala, J. C. Maerz, and L.K. Belden.

2.1 ABSTRACT

Parasites vary widely in host specificity. Many trematodes are able to use a variety of intermediate hosts but distribution among these hosts is uneven. Infection among potential hosts may be a result of ecological and environmental factors rather than a preference for a specific host. *Metagonimoides oregonensis* is a digenetic trematode that uses plethodontid salamanders as second intermediate hosts in the Eastern U.S. Infection intensity is quantifiable in salamanders because the trematode encysts in salamander muscle tissue that can be cleared and stained to allow observation directly through the skin. The goal of our study was to identify which stream salamander species are capable of serving as second intermediate hosts for *M. oregonensis*. We surveyed 289 salamanders from 23 Appalachian headwater sites in North Carolina. Six plethodontid species were collected in the survey including *Desmognathus quadramaculatus* (n=69), *Eurycea wilderae* (n=160), *D. ocoee* (n=31), *D. monticola* (n=3), *E. guttolineata* (n=7), and *Gyrinophilus porphyriticus* (n=19). We found infection in all species except *D. monticola*. We then focused our analysis on comparing infection in *D. quadramaculatus* with *E. wilderae*, the species for which we had robust sample sizes. We found *D. quadramaculatus* had significantly higher infection prevalence and intensity, probably because *D. quadramaculatus* has a longer aquatic larval period and thus greater cumulative exposure to the parasite.

2.2 INTRODUCTION

All animals serve as hosts to a diverse array of parasites; however, parasites are rarely evenly distributed among species or individuals within a host community. Although parasites generally favor one host species, they are often capable of infecting multiple host species (Evans 1983). Ecological and environmental factors may result in an uneven distribution of a parasite among these potential host species (Christensen et al. 1980, Poulin 2005, Holland 2010, Lagrue 2011, Wojdak et al. 2013). Moreover, within a host population a few individuals tend to have many parasites while most individuals have very few (Dobson and Shaw 1995, Poulin 2007). This variation often leads to parasite infection intensity data from natural populations fitting a negative binomial distribution, indicating infection is likely not a result of random host-parasite encounters.

There has been substantial debate about the factors that cause these non-normal distributions, but differential exposure, variation in host susceptibility, parasite selection of preferred hosts, and host condition may all contribute to this pattern (Morrill 2012). Susceptibility to infection may not be related to taxonomic similarity among species (Detwiler and Janovy 2008), indicating that parasites are largely opportunistic and infection of different hosts may be more dependent on host ecological similarities, such as habitat use and diet (Poulin 2005). Parasite distribution can be contingent upon host susceptibility to infection, which may vary depending on host physiology and behavior. Larger species may be more susceptible to infection simply due to increased mass available for infection (Detwiler and Janovy 2008), and life history characteristics and habitat selection may make some hosts more readily available to parasitism (Goater et al. 1987). Organisms may also employ a variety of behaviors to limit infection by parasites including grooming, migration, avoidance of areas with high densities of parasites, and eliminative behaviors (Daly and Johnson 2010). For example, many amphibians

exhibit defensive behaviors, such as exaggerated or increased swimming, in the presence of trematodes to minimize or avoid infection (Thiemann and Wassersug 2000, Johnson et al. 2001, Taylor et al. 2004, Daly and Johnson 2010, Koprivnikar et al. 2012); however, species vary in their effectiveness at avoiding infection (Taylor et al. 2004). Finally, community composition and relative abundance of available hosts and non-hosts can also be important in the distribution of infection among host species (Johnson et al. 2012), because differences in composition may influence the encounter rates of parasites with potential hosts.

Digenetic trematodes are common parasites in aquatic systems. They typically have complex life cycles involving trophic links, where transmission relies on consumption of one host by the next host in the life cycle. In recent years, several studies have examined the effects of amphibian community structure on the distribution of infection levels by *Riberoia ondatrae* and *Echinostoma trivolvis* among potential hosts (Johnson et al. 2008, Johnson and Hartson 2009, Raffel et al. 2010, Rohr et al. 2010, Belden and Wojdak 2011, Johnson et.al. 2012). However, outside of those systems, little is known about biotic factors that influence parasite distribution among host amphibians.

Metagonimoides oregonensis is a Heterophyid trematode associated with freshwater streams. Adult *M. oregonensis* live and sexually reproduce primarily in the small intestine of raccoons, *Procyon lotor* (Price 1931, Sawyer 1958, Schaffer et al. 1961, Harkema and Miller 1964, Bafundo et al. 1980). Eggs are shed with the raccoon feces. If they land in a stream, miracidia infect the first intermediate host, *Elimia proxima (=Pleurocera proxima),* a pleurocerid snail. It is unknown if infection occurs via penetration of the snail by miracidia or consumption of the miracidia by the snail (Burns and Pratt 1953). The miracidia migrate to the digestive glands of the snail where they develop into rediae, which in turn produce cercariae. From this

point, the cercariae can either encyst in the snail as metacercariae or leave the snail to infect another host; infection of a distinct second intermediate host seems to vary across the range of the parasite (Burns and Pratt 1953, Lang and Gleason 1967). In North Carolina, stream-dwelling plethodontid salamanders (e.g. *Desmognathus* spp., *Eurycea* spp.) seem to be common second intermediate hosts (Goater et al. 1987, Belden et al. 2012). After making contact with the host, the cercariae penetrate the skin and encyst as metacercariae in striated muscle tissue. These cysts remain dormant until a raccoon consumes the host (Burns and Pratt 1953).

The southern Appalachian region is a global hotspot for stream salamander diversity where as many as eight species routinely occur within a single stream reach. This results in a large number of potential second intermediate host species for *M. oregonensis*; however, salamander species vary dramatically in abundance, larval period, and size. Therefore *M. oregonensis* prevalence may vary among species. In his survey of North Carolina salamanders, Rankin (1937) found unknown metacercariae in the muscle tissue of *Desmognathus fuscus* and *Desmognathus quadramaculatus*, which appear consistent with a *M. oregonensis* infection. Burns and Pratt (1953) were the first to experimentally complete the life cycle of *M. oregonensis* by infecting several ranid frog species with cercariae. Lang and Gleason (1967) also experimentally infected a variety of amphibians with cercariae, including *D. fuscus,* and multiple anurans. Although *M. oregonensis* appears capable of infecting anurans, anurans are far less common than salamanders in Appalachian streams. Goater et al. (1987) conducted a later survey of North Carolina salamanders and found *M. oregonensis* metacercariae in the musculature of *D. quadramaculatus* and *D. marmoratus*, although they did not quantify the infection intensity. Belden et al. (2012) found infection levels ranging from 53-687 metacercariae in larval *D. quadramaculatus* (13-26 mm SVL) collected in North Carolina. To date, no study has been

published comparing the relative prevalence of *M. oregonensis* among salamander species. The goal of the current study was to examine multiple salamander species common to the southern Appalachian region to determine which species are naturally infected, and whether there are differences in prevalence or intensity of infection among the host species. We predicted that life history, specifically the length of the aquatic larval period, would play a strong role in determining infection prevalence and intensity for specific hosts.

2.3 MATERIALS AND METHODS

Field collections

As part of a larger study (described in Cecala 2012, Webster et al. 2012), stream salamanders were collected from 31 sites within the upper Little Tennessee River basin in North Carolina in 2009. At each site, a 150 m reach was identified upstream of nearby road crossings. Within the reach 1 m^2 plots were created every 5 m. Each plot contained a 25 x 40 cm leaf litter bag constructed from 1 cm^2 plastic mesh and filled with leaf litter from the stream banks or the nearest upstream source. After 48 hours the bags were removed from the stream, placed in a bucket, and gently agitated in water to free salamanders. The water, debris, and salamanders were then poured through a net to separate out the salamanders. After the leaf litter bags were removed, each plot was actively searched by turning cobble and dip-netting the area. Each plot was resampled over 3 consecutive days, and on days 1 and 2, individuals were identified and released. On day 3 individuals were collected, euthanized with an overdose of MS-222 (tricaine methanesulfonate), fixed in 10% buffered formalin and stored in 35% ethanol. Specimens were transferred to 70% ethanol prior to clearing and staining to determine infection intensity. Also in 2009, snail first intermediate hosts were collected at five of the sites where salamanders were collected. Between 45 and 55 *Elimia proxima* were haphazardly collected within a 50 m reach at

each location. These sites included (with snail and salamander sample sizes in parentheses): Howard Branch (n_{snail}=50, n_{sal}=6), Upper Caler Creek (n_{snail}=55, n_{sal}=5), Jaycee Park (n_{sanil}=45, n_{sal}=11), Upper Skeenah Creek (n_{snail}=55, n_{sal}=11) and Jones Creek (n_{snail}=50, n_{sal}=20). Snails were brought to the laboratory and dissected to determine infection status. If first intermediate infection was found, cercariae were wet mounted on slides and identified based on morphology (Burns and Pratt 1953, Schell 1985).

Salamander parasite estimation

We used a two-stage process to estimate *M. oregonensis* prevalence and intensity among salamanders following Belden et al. (2012). First, we examined each individual under a dissecting scope for metacercariae. For the purpose of this study we examined only the area of the abdomen between the fore and hind legs. We counted all visible metacercariae and photographed each salamander's abdomen. We then cleared and stained the animals using a modified version of the protocol of Hanken and Wassersug (1981) as follows. We transferred each specimen to a 50 mL centrifuge tube containing 0.01% Alcian blue cartilage stain for 24 h. Next, we transferred each specimen to EtoH:Acetic Acid (50:50) for 24 h, followed by 100% ethanol for 24 h, then we soaked each specimen in tapwater for 24 h and transferred to 1% trypsin in 30% saturated sodium borate solution to clear the tissues. Specimens were left in this solution until they were limp and blue cartilage stain was visible (24 h - 96 h). Next, we transferred the specimens to Alcian red in 0.5% KOH for 24 h to stain bones and trematodes within the cysts red. Specimens were then moved through two series of 24 h 0.5% KOH rinses before a four part series of KOH:glycerin stepdowns, from 2:1, to 1:1, to 1:2 to 100% glycerin. For the larger, more darkly pigmented individuals, we added 2-4 drops of hydrogen peroxide to the 2:1 KOH: glycerin solution to help bleach the pigment. We stored specimens in 100%

glycerin. After clearing and staining, we counted all metacercariae that were visible from the ventral side bounded by the four limbs under a dissecting microscope, and photographed each specimen.

Statistical Analysis

Because of limited sample sizes for four of the six species (*D. monticola, D. ocoee, G. porphyriticus,* and *E. guttolineata*), we focused our statistical comparisons only on the two most abundant species: *D. quadramaculatus* (n=69) and *E. wilderae* (n=160). We compared infection prevalence between the two species using a chi square test. To predict the number of metacercariae in an individual (infection intensity), we used a generalized linear model with a negative binomial error distribution and an identity link, with species as a categorical factor (*D. quadramaculatus* or *E. wilderae*) and snout-vent length as a continuous covariate. We chose an identity link based on biological grounds because we expect salamanders to accrue parasites at a constant rate throughout their larval periods. Comparing the AIC of an identity link model with the log link function also supported the use of the identity link. Metacercariae counts of four *D. quadramaculatus* salamanders were an order of magnitude higher than the rest and were excluded from the analysis despite their possible biological importance. No model accounted for these four points and still offered a reasonable description of the data. We analyzed the data without those points and with the caveat that very few individuals will get very high infections, as typical of an aggregated parasite infection model. We also qualitatively compared general patterns of snail infection prevalence to total salamander infection prevalence (= all individuals of all species present) among the five sites where those data were available. Statistical analyses were not performed on this data because of the low sample size of sites.

2.4 RESULTS

We examined 289 salamanders representing six species from the 31 sites sampled in North Carolina. Individuals from eight of the sites were eliminated from further consideration because the parasites appeared to be absent based on preliminary observations for visible metacercariae (Belden et al. 2012). Six species were represented among the 23 sites: *Desmognathus quadramaculatus* (n=69), *Eurycea wilderae* (n=160), *D. monticola* (n=3), *D. ocoee* (n=31), *Gyrinophilus porphyriticus* (n=19), *and E. guttolineata* (n=7). These sample sizes roughly mimic natural relative abundances, with *Desmognathus quadramaculatus* and *Eurycea wilderae* as the most abundant species (Milanovich 2010).

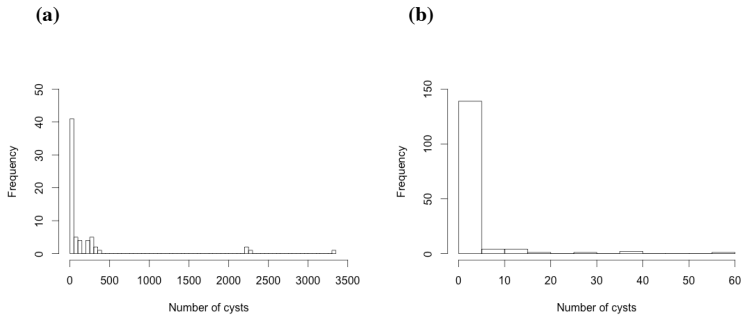
Table 2.1 Prevalence and intensity of infection for the six salamander species collected, in decreasing order of sample size.

Species	Infected/Total (Prevalence)	Range Intensity (median)
Eurycea wilderae	23/160 (14.4)	1-59 (7)
Desmognathus quadramaculatus	33/69 (47.8)	1-3321 (133)
Desmognathus ocoee	7/31 (22.5)	1-21 (4)
Gyrinophilus porphyriticus	1/19 (5.2)	136 (-)
Eurycea guttolineata	2/7 (28.6)	8-13 (10.5)
Desmognathus monticola	0/3 (0)	- (-)

Metagonimoides oregonensis infection was found in five of the six species of salamanders (in all species except *D. monticola*; Table 2.1). Infection prevalence was highly variable among all species and ranged from approximately 5-48%. Infection prevalence differed between *E. wilderae* (14.4%) and *D. quadramaculatus* (47.8%) ((χ^2=29.2, p<0.0001). We compared the distribution of infection intensities among individual *E. wilderae* and *D. quadramaculatus* because they were the two most abundant species. As expected, the parasite distributions were aggregated, in which a few individuals carried the majority of infection (Figure 2.1). Individual infection intensity varied among species, ranging from a median of 4

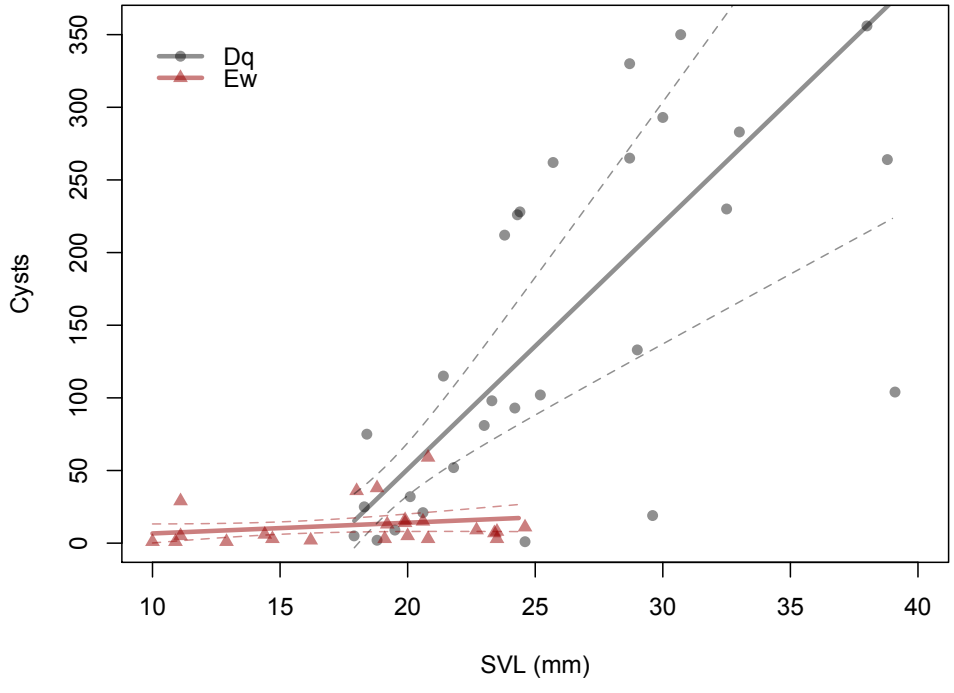
metacercariae (*D. ocoee)* to 133 metacercariae *(D. quadramaculatus)*. Median infection intensity for *D. quadramaculatus* (median=133 metacercariae) was approximately 19x greater than infection intensity for *E.* wilderae (median = 7 metacercariae).

Figure 2.1 Distribution of *M. oregonensis* infection among sites for (a) *Desmognathus quadramaculatus* and (b) *Eurycea wilderae*. Note scale difference between species.



Metacercariae intensity increased with increasing body size (snout-vent length), and depended on species (parameter estimate=(-16.2±3.8, p<0.0001, Figure 2.2), but the rate of parasite accrual differed among salamander species (parameter estimate=287.4±73.1, p<0.0001, Figure 2.2). *Desmognathus quadramaculatus* had more parasites in general and had a much greater rate of parasite accrual (as illustrated by the steeper slope in Figure 2.2).

Figure 2.2 The number of metacercariae found in *D. quadramaculatus* (circle) and *E. wilderae* (triangle). Points represent individual animals, thick lines are model predictions and the dashed lines are 95% confidence intervals around the model predictions.



Elimia proxima snails were collected at five of the 23 salamander collection sites. Snail infection prevalence with *M. oregonensis* ranged from 2% to 87% with a median of 7%. At one site, one snail (2% prevalence) was co-infected with a virgulate-type trematode (Schell 1985), and at another site there was one snail (2% prevalence) infected with what was likely *Sanguinicola fontinalis* (Hoffman et al. 1985). No analysis was performed because data were only available for five sites. However, at most sites snail infection prevalence was low (<10%) and salamander infection prevalence at the corresponding site was either similar or higher. Only one site had high snail infection prevalence (87%) and correspondingly high salamander infection prevalence (80%). This suggests snail and salamander infection prevalence may be correlated, but more data will be needed to validate that conclusion.

2.5 DISCUSSION

Metagonimoides oregonensis metacercariae were present in five of the six salamander species we collected. Although we did not detect any infection in *D. monticola*, that is likely the result of a small sample size (n=3). It seems improbable that *M. oregonensis* cannot infect *D. monticola* given that we detected the parasite in other *Desmognathus* spp. as well as distantly related confamilial species. While our results do indicate that *M. oregonensis* can infect a variety of stream salamander species, our data suggest that *D. quadramaculatus* might be particularly important in transmission dynamics of this parasite given the higher prevalence and intensity of infection found in that species.

Several factors might account for the variation among species in infection prevalence and intensity. Life history traits, and in particular the length of the larval period and thus time spent in the stream following metamorphosis could influence the intensity of infection. All of the

salamander species we examined have an aquatic larval period, but it varies in duration. Of the species we examined, *D. quadramaculatus* has the longest larval period, lasting 36-48 months, while *E. wilderae* has a larval period of 12-24 months. Longer larval periods may result in a higher intensity of infection because of increased exposure time to snails that are shedding parasites. *Desmognathus ocoee* (9-10 mos.), *D. monticola* (8-13 mos.), and *E. guttolineata* (5-16 mos.) all have much shorter larval periods, which may explain the relatively low intensity of infection in our samples of those species. The one *G. porphyriticus* larva that was infected was the only other larva to have infection intensity (133 metacercariae) comparable to the highly infected *D. quadramaculatus*. Similar to *D. quadramaculatus, G. porphyriticus* has a 36-60 month larval period. Of course, we must qualify our conclusions with the fact that we had small sample sizes for all but two species. The low numbers of *D. monticola, D. ocoee, E. guttolineata, and G. porphyriticus* larvae in our samples, however, may also indicate that these species' larval stages are less abundant in these streams and so are relatively rare targets for *M. oregonensis* compared with *D. quadramaculatus* and *E. wilderae*. In part, the role of relatively rare hosts in transmission dynamics may therefore depend on whether the two more abundant species in the system are replaced/displaced by the presence of rare species or whether the overall number of salamanders is increased with rare species, and therefore increase total host availability for the parasite, as discussed in Wojdak et al. (2013).

Further evidence suggesting that life history differences are critical for infection is provided by the relationship between infection and body size. Body size as measured by snout-vent length (SVL) correlates strongly with age in salamanders (Bruce 2002). When excluding the four individuals with over 2000 metacercariae, we found a positive relationship between body size and intensity of infection for *D. quadramaculatus*, which ranged in size from approximately

14 to 39 mm. Again, this may indicate that individuals are, in general, accumulating metacercariae over time, such that larger, older individuals, who have been exposed for longer periods, have higher numbers of metacercariae. Larger size classes are lacking for *E. wilderae* because they usually metamorphose into a more terrestrial adult after a single larval season. Since their exposure time is limited by the short larval period, their infection level may be more dependent on environmental factors, and less on age or size.

Desmognathus quadramaculatus adults tend to stay in the streams following metamorphosis where they commonly burrow into the stream banks (Petranka 1998). In contrast, *E. wilderae* become more terrestrial as adults and disperse to the forest floor. As a result of these differences in life history, *D. quadramaculatus* may be exposed at all life stages whereas exposure of *E. wilderae* may be restricted to the relatively brief aquatic larval stage.

Prevalence of snail infection among sites is also anticipated to vary widely based on a suite of ecological and environmental factors. Indeed, at the five sites described herein, snail infection prevalence ranged 3-87%. Johnson and Chase (2004) showed that amphibian trematode infection in ponds tends to increase as snail biomass in a system increases. Therefore, snail density may be a better indicator of salamander infection than snail infection prevalence *per se*, although both factors are likely to influence the risk of infection for a salamander host. Indeed, high prevalence in snails in a system with very few snails may be a low risk environment for salamanders, while even relatively low prevalence may result in a high risk of salamander infection if snail density is very high.

Variability in infection level among potential hosts may have strong implications for transmission dynamics. Raccoons, the definitive host for this system, are nocturnal generalist mesopredators that prefer to forage along forest edges and in wetlands, especially during the

early spring and summer (Barding 2008). They tend to forage using area-restricted searches and focus most of their efforts in areas with shallow water and along linear stream and habitat boundaries (Byrne 2012). Crayfish are a common and abundant prey item for raccoons in this habitat (Baker et al. 1945). Plethodontid salamanders are also abundant and are found in the same microhabitats in streams as crayfish. Because *D. quadramaculatus* is one of the most abundant salamander species and represents the largest portion of salamander biomass in the study streams (Milanovich 2010), they may also be relatively common prey for raccoons. If *D. quadramaculatus* is the primary second intermediate host in the transmission cycle, then variation in the abundance of *D. quadramaculatus* may be an important determinant of *M. oregonensis* distribution in the landscape. Other studies have linked secondary or definitive host abundance to the prevalence and persistence of trematodes in first intermediate host snails (Lafferty 1997, Byers et al. 2010). *Desmognathus quadramaculatus* abundance does vary spatially, even among fully forested watersheds (Cecala 2012), which could drive spatial patterns in parasite infection.

2.6 REFERENCES

Bafundo, K. W., W. E. Wilhelm, and M. L. Kennedy. 1980. Geographic variation in helminth parasites from the digestive tract of Tennessee raccoons, *Procyon lotor*. The Journal of Parasitology **66**: 134-139.

Baker, R. H., C. C. Newman, and F. Wilke. 1945. Food habits of the raccoon in eastern Texas. The Journal of Wildlife Management **9**: 45-48.

Barding, E. E., and T. A. Nelson. 2008. Raccoons use habitat edges in northern Illinois. American Midland Naturalist **159**: 394–402.

Belden, L. K., and J. M. Wojdak. 2011. The combined influence of trematode parasites and predatory salamanders on wood frog (*Rana sylvatica*) tadpoles. *Oecologia* **166**: 1077–1086.

Belden, L. K., W. E. Peterman, S. A. Smith, L. R. Brooks, E. F. Benfield, W. P. Black, Z. Yang, and J. M. Wojdak. 2012. *Metagonimoides oregonensis* (Digenea, Heterophyidae) infection in pleurocerid snails and *Desmognathus quadramaculatus* salamander larvae in southern Appalachian streams. The Journal of Parasitology **98**: 760-767.

Bruce, R. C., J. Castanet, and H. Francillon-Vieillot. 2002. Skeletochronological analysis of variation in age structure, body size, and life history in three species of desmognathine salamanders. Herpetologica **58**: 181–193.

Burns, W. C. and I. Pratt. 1953. The life cycle of *Metagonimoides oregonensis* Price (Trematoda: Heterophyidae). The Journal of Parasitology **39**: 60-69.

Byers, J. E., I. Altman, A. M. Grosse, T. C. Huspeni, J. C. Maerz. 2010. Using parasitic trematode larvae to quantify an elusive vertebrate host. Conservation Biology **25**: 85-93.

Byrne, M. E., and M. J. Chamberlain. 2012. Using first-passage time to link behaviour and habitat in foraging paths of a terrestrial predator, the racoon. Animal Behaviour **84**: 593–601

Cecala, K.K. 2012. The role of behavior in influencing headwater salamander responses to disturbance. Ph.D. Dissertation. University of Georgia, Athens, Georgia. 155 p.

Christensen, N., F. Frandsen, and M. Z. Roushdy. 1980. The influence of environmental conditions and parasite-intermediate host-related factors on the transmission of *Echinostoma liei*. Zeitschrift For Parasitenkunde Parasitology Research **64**: 47–63.

Daly, E. W. and P. T. Johnson. 2010. Beyond immunity: Quantifying the effects of host anti-parasite behavior on parasite transmission. Oecologia **165**: 1043–1050.

Detwiler, J. and J. Janovy. 2008. The role of phylogeny and ecology in experimental host

specificity: Insights from a eugregarine–host system. The Journal of Parasitology **94**: 7-12.

Evans, N. A., and D. M. Gordon. 1983. Experimental observations on the specificity of *Echinoparyphium recurvatum* toward second intermediate hosts. Parasitology Research **69**: 217–222.

Goater, T. M., G. W. Esch, and A. O. Bush. 1987. Helminth parasites of sympatric salamanders: Ecological concepts at infracommunity, component and compound community levels. American Midland Naturalist **118**: 289-300.

Hanken, J. and R. Wassersug. 1981. The visible skeleton. Functional Photography **16**: 22-44.

Harkema, R. and G. C. Miller. 1964. Helminth parasites of the raccoon, *Procyon lotor* in the southeastern United States. The Journal of Parasitology **50**: 60-66.

Hoffman, G. L., B. Fried, and J. E. Harvey. 1985. *Sanguinicola fontinalis* sp nov. (Digenea: Sanguinicolidae): a blood parasite of brook trout, *Salvelinus fontinalis* (Mitchill), and longnose dace, *Rhinichthys cataractae* (Valenciennes). Journal of Fish Diseases **8**: 529–538.

Holland, M. P. 2010. Echinostome-induced mortality varies across amphibian species in the field. The Journal of Parasitology **96**: 851–855.

Johnson, P. T. J., K. B. Lunde, R. W. Haight, J. Bowerman, and A. R. Blaustein. 2001. *Ribeiroia ondatrae* (Trematoda: Digenea) infection induces severe limb malformations in western toads *Bufo boreas*." Canadian Journal of Zoology **79**: 370–379.

Johnson, P. T. J., and J. M. Chase. 2004. Parasites in the food web: Linking amphibian malformations and aquatic eutrophication. Ecology Letters **7**: 521–526.

Johnson, P. T. J., R. B. Hartson, D. J. Larson, and D. R. Sutherland. 2008. Diversity and disease: Community structure drives parasite transmission and host fitness. Ecology Letters **11**: 1017–1026.

Johnson, P. T. J., and R. B. Hartson. 2009. All hosts are not equal: Explaining differential patterns of malformations in an amphibian community. Journal of Animal Ecology **78**: 191–201.

Johnson, P. T. J, J. R. Rohr, J. T. Hoverman, E. Kellermanns, J. Bowerman, and K. B. Lunde. 2012. Living fast and dying of infection: Host life history drives interspecific variation in infection and disease risk." Ecology Letters **15**: 235–242.

Koprivnikar, J., Gibson, C.H., and Redfern, J.C. 2012. Infectious personalities: Behavioural syndromes and disease risk in larval amphibians. Proc. R. Soc. B **279**: 1544–1550.

Lafferty, K.D. 1997. Environmental parasitology: What can parasites tell us about human impacts on the environment? Parasitology Today **13**: 251–255.

Lagrue, C., D. W. Kelly, A. Hicks, and R. Poulin. 2011. Factors influencing infection patterns of trophically transmitted parasites among a fish community: host diet, host–parasite compatibility or both? Journal of Fish Biology **79**: 466–485.

Lang, B. Z. and L. N. Gleason. 1967. Life cycle of *Metagonimoides oregonensis* Price, 1931 (Trematoda: Heterophyidae) in North Carolina. The Journal of Parasitology **53**: 93.

Milanovich, J. R. 2010. Modeling the current and future roles of stream salamanders in headwater streams. PhD dissertation. University of Georgia, Athens, Georgia. 164 p.

Morrill, A., and M. R. Forbes. 2012. Random parasite encounters coupled with condition-linked immunity of hosts generate parasite aggregation. International Journal for Parasitology **42:** 701–706.

Petranka, J. W. 1998. Salamanders of the United States and Canada 1st ed. Smithsonian Books, Washington, D.C., 587 p.

Poulin, R. 2005. Relative infection levels and taxonomic distances among the host species used by a parasite: Insights into parasite specialization. Parasitology **130**: 109–115.

Poulin, R. 2007. Are there general laws in parasite ecology? Parasitology **134**: 763-776.

Price, E. W. 1931. *Metagonimoides oregonensis*, a new trematode from a raccoon. Journal of the Washington Academy of Sciences **21**: 405-407.

Raffel, T. R., J. T. Hoverman, N. T. Halstead, P. J. Michel, and J. R. Rohr. 2010. Parasitism in a community context: Trait-mediated interactions with competition and predation. Ecology **91**: 1900–1907.

Rohr, J. R., T. R. Raffel, and C. A. Hall. 2010. Developmental variation in resistance and tolerance in a multi-host–parasite system. Functional Ecology **24**: 1110–1121.

Rankin, J. S. 1937. An ecological study of parasites of some North Carolina salamanders. Ecological Monographs **7**: 169-269.

Sawyer, T. K. 1958. *Metagonimoides oregonensis* Price, 1931 from a Georgia Raccoon with a Note on *Sellacotyle mustelae* Wallace, 1935. The Journal of Parasitology **44**: 63.

Schaffer, G. D., W. R. Davidso, V. F. Nettles, E. A. Rollor. 1961. Helminth parasites of translocated raccoons (*Procyon lotor*) in the southeastern United States. Journal of Wildlife Diseases **17**: 217-227.

Schell, S. C. 1985. Trematodes of North America. University of Idaho Press, Moscow, Idaho: 263 pages.

Shaw, D.J., and Dobson, A.P. 1995. Patterns of macroparasite abundance and aggregation in wildlife populations: A quantitative review. Parasitology **111**: S111–S133.

Taylor, C. N., K. L. Oseen, and R. J. Wassersug. 2004. On the behavioural response of *Rana* and *Bufo* tadpoles to Echinostomatoid cercariae: Implications to synergistic factors influencing trematode infections in anurans. Canadian Journal of Zoology **82**: 701–706.

Thiemann, G.W., and Wassersug, R.J. 2000. Patterns and consequences of behavioural

responses to predators and parasites in Rana tadpoles. Biological Journal of the Linnean Society **71**: 513–528.

Webster, J.R., E.F. Benfield, K. Cecala, J.F. Chamblee, C. Dehering, T.Gragson, J. Hepinstall, C.R. Jackson, J. Knoepp, D. Leigh, J. Maerz, C. Pringle, and H.M. Valett. 2012. Water quality and exurbanization in southern Appalachian streams. Pages 91-106 in P.J. Boon and P.J. Raven (editors). River Conservation and Management. Wiley-Blackwell, Chichester, UK.

Wojdak, J. M., L. Clay, S. Moore, T. Williams, L. K. Belden. 2013. *Echinostoma trivolvis* (Digenea: Echinostomatidae) second intermediate host preference matches host suitability. Parasitology Research **112**:799–805

3. EFFECTS OF *METAGONIMOIDES OREGONENSIS* EXPOSURE ON AMPHIBIAN LOCOMOTOR PERFORMANCE

J. A. Wyderko, I. Cedillos, L. K. Belden

3.1 ABSTRACT

Parasitic infection often results in morphological or behavioral alterations to the host. In particular, trophically transmitted parasites frequently alter their secondary intermediate host's behavior, either directly or indirectly, to increase the likelihood that it will be consumed by the definitive host. *Metagonimoides oregonensis* is a trematode that infects raccoons as definitive hosts, the snail *Elimia proxima* as a primary intermediate host, and encysts as metacercariae in the muscle tissue of the secondary intermediate host, usually plethodontid salamanders. We tested locomotor behavior of *Hyla versicolor* (Gray Tree Frog) tadpoles, *Desmognathus quadramaculatus* (Black-bellied Salamander) and *Eurycea wilderae* (Two-lined salamander) larvae following cercariae exposure to determine whether *M. oregonensis* infection had any negative effects on swimming ability that may make the host more susceptible to capture by raccoons. We performed locomotor trials on animals one and seven weeks after parasite exposure. We found no significant effects of parasitic infection on locomotor behavior for tadpoles, *D. quadramaculatus,* or *E. wilderae* salamanders.

3.2 INTRODUCTION

Predators frequently remove highly parasitized individuals from populations, usually because these individuals are more conspicuous to predators based on morphological, behavioral or physiological changes that occur as a result of being parasitized (Hudson et al. 1992, Poulin 1994, Packer et al. 2003, Duffy et al. 2005). Highly parasitized prey may be a more profitable choice for a predator if they are easier to find or consume (Temple 1987). Parasites that are

trophically transmitted and cause alterations to their host that increase the odds they will be consumed will be favored by natural selection (Moore 1995). Host manipulation by parasites may be a direct or an indirect effect of infection. For example, the trematode *Euhaplorchis californiensis* encysts in the brain of the Pacific killifish (*Fundulus parvipinnis*) and alters locomotor performance so that their swimming patterns are more conspicuous to avian predators (Lafferty and Morris 1996). In contrast, indirect effects on hosts may occur as a by-product of infection or pathology. For example, increased metabolic oxygen demand caused by a parasite may result in fish swimming near the surface of the water more frequently (Giles 1983). Whether direct or indirect, these behavioral modifications are only evolutionarily advantageous to the parasite in cases where modifications result in increased predation by the correct definitive host.

Trematodes are a class of flatworms with complex life cycles, usually involving at least one trophic link. Adult worms sexually reproduce within the vertebrate definitive host. Eggs are shed with the feces into the environment where they hatch into miracidia. The miracidia generally then infect a specific molluscan first intermediate host species. Within the mollusk the parasite undergoes several asexual developmental stages (i.e. rediae, cercariae). When mature, cercariae leave the mollusk and enter the environment in search of a second intermediate host. Second intermediate hosts are highly variable across trematodes and can include amphibians, mollusks, or direct encystment in the environment, for example on vegetation. Once cercariae encounter an appropriate host, they penetrate and encyst to form metacercariae. The metacercariae remain dormant until the second intermediate host is consumed by the definitive host, at which point the metacercariae excyst and develop into adult worms. For the parasite to continue to propagate, the second intermediate host must be consumed by the definitive host.

Therefore, it may be evolutionarily favorable for the parasite to alter the second intermediate host in a way that makes them more susceptible to predation. There has been some speculation that the trematode *Riberoia ondatrae* induces intensity-dependent limb malformations in tadpoles in order to increase susceptibility to predation, but no research has yet confirmed this (Johnson et al. 2004). There has been relatively little direct study of behavioral manipulation of amphibian hosts by trematode parasites.

Metagonimoides oregonensis is a trematode common to streams in the Appalachian region. Raccoons are the definitive host in this region. The gastropod *Elimia (=Goniobasis) proxima* is the first intermediate host and a variety of amphibians serve as second intermediate, depending on the region. In studies on the west coast of the United States, at least three species of anurans appear capable of serving as second intermediate hosts; Burns and Pratt (1953) successfully infected *Rana aurora, R. sylvatica,* and *Lithobates catesbeianus (=R. catesbeianus)* in the lab. In the southern Appalachian region, metacercariae encyst in the muscle tissue of a variety of plethodontid stream salamanders. *Desmognathus quadramaculatus* and *Eurycea wilderae* both appear to be commonly infected species (Wyderko et al. *in review*). In general, *D. quadramaculatus* appear to be the more common host of *M. oregonensis*, probably because they have relatively lengthy larval periods in the streams and remain primarily aquatic as adults.

One possible mechanism for increased susceptibility of larval amphibians to predation is a decreased ability to escape quickly from an attack due to decreased muscle response and a decrease in locomotor performance. This seems likely in this system because *M. oregonensis* metacercariae encyst in the muscle tissue. The goal of this study was to evaluate the effects of *M. oregonensis* exposure on larval *Hyla versicolor* (gray treefrog), *Eurycea wilderae cirregae* (Two-lined salamander), and *Desmognathus quadramaculatus* (Black-bellied salamander)

locomotion. *Hyla versicolor* tadpoles were examined because they typically have low trematode infection and minimal pathology when exposed to another, usually highly pathogenic trematode *Riberoia ondatrae* (Johnson and Hartson 2009). The two salamanders were chosen because previous work has shown they are common second intermediate hosts of *M. oregonensis* in this region (Wyderko et al. *in review*, Belden et al. 2012). We expected both tadpoles and salamanders to have reduced locomotor function with increased trematode exposure as a result of metacercariae encysting in muscle tissue.

3.3 MATERIALS AND METHODS

Amphibian Rearing

A 1000 L mesocosm was established as a *H. versicolor* tadpole stock tank outdoors at Virginia Tech's Kentland farm (Montgomery County, Virginia) in May 2012. Naturally laid *H. versicolor* eggs were added to the mesocosm to provide a source of parasite-free tadpoles. After hatching, 64 tadpoles were returned to the laboratory for use in exposure trials. Prior to infection, tadpoles were kept at room temperature (~ 21°C), in aquaria filled with dechloraminated tap water. Windows in the room provided natural daylight, and tadpoles were fed a mix of fish flakes and ground rabbit chow every two or three days. Sixteen tadpoles were weighed for an estimate of initial mass (0.029 g ± 0.005) and five tadpoles were examined for an estimate of developmental stage (all were at Gosner stage 25; Gosner 1960).

Larval *D. quadramaculatus* (n=30) and larval *E. wilderae* (n=15) were collected from a stream (Lick Run) in Montgomery County, Virginia. Salamanders were identified via morphological characteristics in the field (Petranka 1998). No *Elimia proxima* were present at this site, so salamanders were assumed to be uninfected with *M. oregonensis*. Salamanders were collected 48 hours prior to exposure and were housed in an 18˚C room on a 12:12 light: dark

cycle. Salamanders were housed in individual 150 mL plastic cups filled with 50 mL decholoraminated water prior to exposure. Salamanders were not weighed prior to the trial to avoid unnecessary stress.

Parasite Exposure

For parasite exposures, tadpoles and salamanders were placed in individual 120 mL cups filled with 50 mL of dechloraminated water. Each individual was randomly assigned to one of three exposure treatment groups: 0, 20, 60 cercariae (n=16 tadpoles/treatment, 30 salamanders/treatment). These groups were then divided in half with one group examined one week post-exposure and the other group examined at seven weeks post-exposure, except for *E. wilderae*. This resulted in eight tadpoles/treatment in each post-exposure sampling point and five *D. quadramaculatus*/treatment. *Eurycea wilderae* were only sampled at seven weeks post-exposure due to small sample size (n=15). We chose these exposure doses based on previous field data (median metacercariae density of *Desmognathus quadramaculatus* =133 and *Eurycea wilderae*=7,Wyderko et al. *In review*). Naturally infected *Desmognathus quadramaculatus* typically have higher intensities of infection than *E. wilderae*, so these doses were relatively high for *E. wilderae* and fairly moderate for *Desmognathus quadramaculatus*.

First intermediate infected *Elimnia proxima* (n=13) were collected from two streams (Rush Fork and Chisholm Creek) in Floyd County, Virginia. They were housed in individual plastic cups in an 11˚C incubator prior to the experiment. To induce shedding of cercariae, snails were removed from the fridge and placed underneath incandescent light bulbs. Cercariae were identified as *M. oregonensis* by morphological characters under a stereomicroscope (Burns and Pratt 1953). Cercariae used in the experiment were less than four hours old. Cercariae were collected with a pipette under a stereomicroscope and transferred to individual wells of a 96-well

plate. Once each well contained the designated number or cercariae, dechloraminated tap water was added to standardize each well's volume and the contents were pipetted into the appropriate cups. Post-exposure tadpoles and salamanders were housed in a room with a 12:12hr dark:light cycle maintained at 18°C, for either one week or seven weeks based on treatment group.

One day after parasite exposure, the water in the 120 mL cups housing the tadpoles was changed. Tadpoles were maintained on their previous diet and the water was changed every two to three days for the duration of the time they were housed in the lab. Salamanders were moved to individual plastic containers (160cm x 270cm x 80cm) with aquarium pebbles and dechloraminated water. They were fed three blackworms (*Lumbriculus variegatus*) every three days, but were not fed 48 hours prior to locomotor trials.

Locomotor Trials

Individual tadpoles were placed in 5.7 L plastic rectangular containers (34.6cm L x 21.0cm W X 12.4cm H) filled to a depth of five cm with 1080 mL of dechloraminated tap water. Tadpoles were allowed to acclimate for one minute and then total activity (defined as time spent doing any movement) was recorded for three minutes. Containers were cleaned and dried between trials.

Salamander locomotor trials were performed in an 18°C cold room during the dark cycle. Each salamander underwent three locomotor trials, separated by 24-hour rest periods. Trials were videotaped using the nightshot setting on a Sony HDR-XR500V video camera. For each trial, salamanders were removed from the plastic container they were housed in by pouring the water through a mesh net. The salamander was then transferred to a plastic cup so they could be placed in a 1 m gutter. The gutter contained 500 mL dechloraminated water at a depth of 0.5 cm. Salamanders were placed in the gutter at the five cm mark. Immediately after being placed in the

gutter, the salamander was gently nudged with the blunt end of a wooden probe. If they did not move forward after five nudges, they were removed from the trial. Salamanders that responded to the nudge and swam down the gutter were allowed to swim until they came to a stop lasting at least five seconds. Salamanders were removed from the gutter using the plastic cup and were placed back into the container they were originally housed in with clean dechloraminated water. Distance traveled was analyzed using iMovie and ImageJ (Abramoff et al. 2004) software.

All animals were euthanized 24-48 hours following the locomotor trial by an overdose of buffered 0.1% MS-222 (Tricaine methanesulfonate). After euthanasia they were transferred to individual 15 mL centrifuge tubes containing 10% formalin. After 24 hours they were transferred to individual tubes containing 70% ethanol and were weighed again. Average post-mortem mass for the one week tadpole group was 0.04 g ± 0.01. Average post-mortem mass for the seven week groups was 0.20 g ± 0.03 for tadpoles, 0.18 g ± 0.09 for *D. quadramaculatus* and 0.12 g ± 0.04 for *E. wilderae*.

Samples were stored in 70% ethanol until clearing and staining could be performed using a modified version of Hanken and Wassersug's protocol (1981, see Wyderko et al. in review) and cysts were quantified.

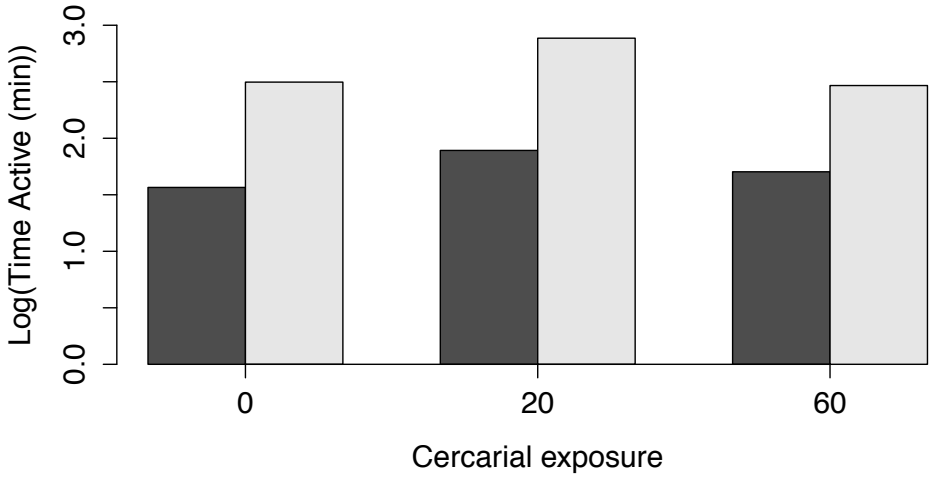
Statistical Analysis

Locomotor performance was compared across the three exposure treatments within each post-exposure group for each individual species, to determine whether level of exposure effected performance. For each one week and seven week group by species, the raw distance traveled (cm) was log-transformed and all groups were found to approximate a normal distribution. An ANOVA was performed for each time point and species comparing distance traveled among the three cercariae treatment groups.

3.4 RESULTS

After clearing and staining, no *M. oregonensis* cysts were visible in any *H. versicolor* individuals at either time point. There were also no significant differences in time active among parasite treatments at one week (F=0.751, p=0.489) or seven weeks (F=0.959, p=0.404) post-exposure (Figure 3.1).

Figure 3.1 Log of time active for *H. versicolor* tadpoles tested 1 week post-exposure (dark grey) and 7 weeks post-exposure (light grey).

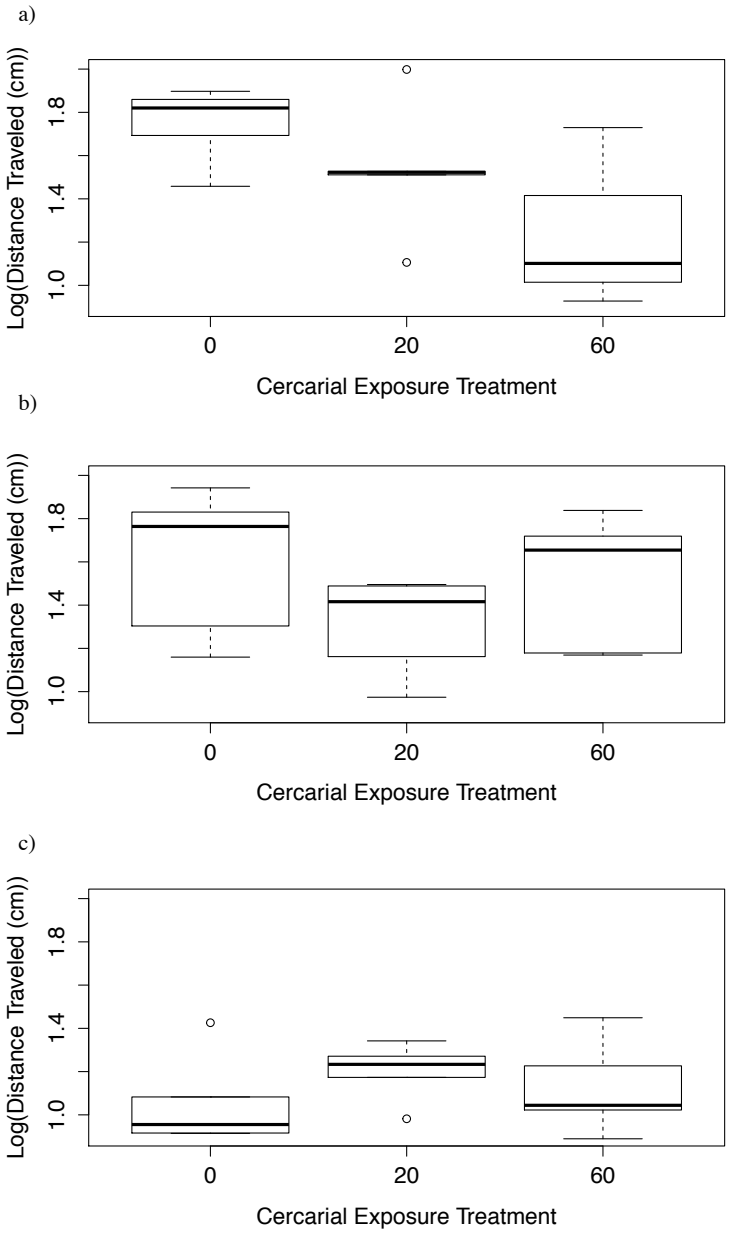


After clearing and staining, some metacercariae were visible in groups exposed to 20 and 60 cercariae for *Desmognathus quadramaculatus* tested one week post-exposure, so we know infection occurred. Metacercariae were extremely immature, and therefore impossible to accurately quantify. *Desmognathus quadramaculatus* salamanders traveled significantly less distance as their exposure level to the parasite increased (F=4.74, p=0.0304) when tested one week after exposure. However, there were two salamanders exposed to 60 cercariae with severe limb malformations that may have confounded the results. These two salamanders were removed and the data were re-analyzed. There were no significant differences in distance

traveled among salamanders in different cercariae treatment groups after the removal of those two individuals ($F=2.595$, $p=0.124$, Figure 3.2a).

After clearing and staining salamanders seven-weeks post-exposure, no metacercariae were visible in either *D. quadramaculatus* or *E. wilderae*. Average distance traveled was not significantly different among cercariae exposure treatments for *D. quadramaculatus* tested seven weeks post-exposure ($F=0.894$, $p=0.437$, Figure 3.2b) or *E. wilderae* tested seven weeks post-exposure ($F=0.668$, $p=0.531$, Figure 3.2c).

Figure 3.2 (a) log of average distance traveled (cm) by *D. quadramaculatus* tested one week post-exposure, for the three cercariae exposure treatments, (b) log of average distance traveled (cm) by *D. quadramaculatus* tested seven weeks post-exposure for the three cercariae exposure treatments (c) log of average distance traveled (cm) by *E. wilderae* tested seven weeks post-exposure for the three cercariae exposure treatments.



3.5 DISCUSSION

We were unable to quantify metacercariae for *D. quadramaculatus* in the one-week test group because they were immature. Only metacercariae that were in superficial muscle tissue were visible. Burns and Pratt (1953) recorded that metacercariae take 45 days to mature and become infective, so finding only immature forms when we euthanized salamander after only 7 days is not surprising. We did not see any metacercariae in any of the seven-week salamanders but we think this was due to problems with the chemicals involved in the clearing and staining process, as we did find immature cysts in *D. quadramaculatus* tested one week after exposure. It is also possible that because salamanders were exposed to relatively low doses of cercariae, that they cleared the infection during the seven week period. The seven-week individuals were cleared and stained at a later date than the tadpoles and one-week post-exposure salamanders.

Exposure to *M. oregonensis* cercariae at the doses examined does not appear to have any effect on larval salamander locomotion. For many parasites negative pathology is intensity-dependent with a threshold level for the induction of measurable pathology (Santos 2011). Previous surveys have shown that larval and juvenile *D. quadramaculatus* can be infected with thousands of metacercariae (Belden et al. 2012, Wyderko et al. in review). Our experimental exposures were much less intense, so infection may have been below the threshold where we would see behavioral effects due to infection. Daily cercariae input into a stream is variable based on prevalence of snail infections but one heavily infected snail can easily release 100+ cercariae per hour (JW *pers. obs.*). Even so, Orlofske et al. (2009, 2013) showed that moderate, gradually accumulated *Echinostoma* infections do not result in significant pathology and have little to no effect on metabolism or fitness of tadpoles. This may be true for *M. oregonensis*, as well. It is likely that salamanders are almost constantly exposed to a low density of cercariae and

so accumulate infections slowly, possibly resulting in different or less pathology than from a more intense, discrete exposure event.

Host factors can play a strong role in susceptibility to infection. Parasites may be unable to penetrate or survive within certain potential hosts due to a host immune response. From the results of this study it appears that *H. versicolor* tadpoles are not susceptible to *M. oregonensis* infection. Johnson and Hartson (2009) found that *H. versicolor* tadpoles experienced very low levels of infection with *Riberoia ondatrae* and had almost no pathology. They speculated that some innate immunity prevented metacercariae establishment since parasites were rapidly destroyed. However, when *H. versicolor* are exposed to high numbers of *Echinostoma sp.* they experience low mortality but still carry heavy metacercariae burdens (Holland 2010). Since we saw no evidence of infection in tadpoles it may be possible that a similar innate immunity applies to *M. oregonensis*, but it may not be an across the board response to trematode infection (Kiesecker and Skelly 2001).

Most of the previous work examining the effects of metacercariae infection on tadpoles has focused on *Riberoia ondatrae* and *Echinostoma spp. Riberoia ondatrae* appears to be fairly pathogenic at high doses and as a result, most of the research has focused on tadpole growth and survival (Johnson 1999, Johnson et al. 2001, Schotthoeffer et al. 2003). *Riberoia ondatrae* also results in severe limb malformations of young tadpoles, presumably making them more vulnerable to predation, however these effects have not been quantified. Echinostomes are very pathogenic to young tadpoles at high exposures (Fried 1997) but at more moderate, continuous doses, they seem to have little effect on tadpole survival (Orlofske et al. 2009). Effect on locomotor performance has not specifically been tested in this system, but Orlofske et al. (2009, 2013) found that infection had no effect on tadpole metabolic oxygen demand or intestine size.

Decreased locomotor performance in salamanders would likely result in increased predation by both hosts (raccoons) and non-hosts (e.g. skunks, snakes) and so may not be evolutionarily favorable for the parasite. However, there has been some speculation that even if host manipulation results in more predation by dead-end hosts as a side effect, the end result may still be more total transmission to the appropriate definitive host than if there was no manipulation at all (Seppala et al. 2005, Seppala et al. 2008, Mouritsen and Poulin 2003, Poulin 2010). Host manipulations may also be specific so as to only increase predation susceptibility to the preferred definitive host predator (Levri 1998, Lagrue et al. 2007). One of the best-studied examples of trematode behavioral manipulation is by *Dicrocoelium dendriticum,* which induces ants to climb grass stalks to encourage consumption by livestock (Spindler 1986). However, this system is unique in that sheep are not usual predators of ants, so strong behavioral manipulation is critical for transmission. For systems where the definitive host is a normal predator of the second intermediate host, empirical evidence showing host manipulation actually results in increased predation by the appropriate host has been limited and largely correlational (Lafferty and Morris 1996, Seppala 2005, Cezilly 2010), so further work is needed exploring these issues.

The results of this study indicate that moderate levels of infection do not have an effect on salamander locomotor performance. It is possible that higher exposures or continuous long-term doses may have different impacts on salamanders. In addition, it is possible that the parasite does not have an impact on host behavior until the metacercariae have matured (Poulin et al. 1994), so it is possible if we had had longer post-exposure time periods we would have seen an effect. In this study we have only tested for effects on locomotor performance, it is possible that infection may have other effects not explored in this study. However, Belden et al. (2012) found no significant pathology associated with metacercariae so it is possible infection does not have

significant negative effects. Other possible effects of infection may include changes in quantity or pattern of foraging behavior, time spent in refuge areas, or response to predatory stimulus.

3.6 REFERENCES

Abramoff, M.D., Magalhaes, P.J., Ram, S.J. 2004. Image processing with ImageJ. Biophotonics International **11**: 36-42.

Belden, L.K., Peterman, W.E., Smith, S.A., Brooks, L.R., Benfield, E.F., Black, W.P., Yang, Z., and Wojdak, J.M. 2012. *Metagonimoides oregonensis* (Heterophyidae: Digenea) infection in pleurocerid snails and *Desmognathus quadramaculatus* salamander larvae in Southern Appalachian streams. Journal of Parasitology **98**: 760–767.

Brodie, E. D. and D. R. Formanowicz Jr. 1987. Antipredator mechanisms of larval anurans: Protection of palatable individuals. Herpetologica **43**: 369-373.

Crowden, A.E., and Broom, D.M. 1980. Effects of the eyefluke, *Diplostomum spathaceum*, on the behaviour of dace (*Leuciscus leuciscus*). Animal Behaviour **28**: 287–294.

Chubb, J.C., Ball, M.A., and Parker, G.A. 2010. Living in intermediate hosts: evolutionary adaptations in larval helminths. Trends in Parasitology **26**: 93–102.

Feder, M.E., and Arnold, S.J. 1982. Anaerobic metabolism and behavior during predatory encounters between snakes (*Thamnophis elegans*) and salamanders (*Plethodon jordani*). Oecologia **53**: 93–97.

Giles, N. 1983. Behavioural effects of the parasite *Schistocephalus solidus* (Cestoda) on an intermediate host, the three-spined stickleback, *Gasterosteus aculeatus*. Animal Behaviour **31**: 1192–1194.

Gosner, K.L. 1960. A simplified table for staging anuran embryos and larvae with notes on identification. Herpetologica **16**: 183–190.

Hanken, J., and Wassersug, R. 1981. The visible skeleton. Functional Photography **16**: 22–44.

Holland, M.P. 2010. Echinostome-Induced Mortality Varies Across Amphibian Species in the Field. Journal of Parasitology **96**: 851–855.

Hudson, P., Dobson, A., and Newborn, D. 1992. Do parasites make prey vulnerable to predation - red grouse and parasites. J. Anim. Ecol. **61**: 681–692.

Johnson, P.T. 1999. The effect of trematode infection on amphibian limb development and survivorship. Science **284**: 802–804.

Johnson, P.T.J., Lunde, K.B., Haight, R.W., Bowerman, J., and Blaustein, A.R. 2001. *Ribeiroia ondatrae* (Trematoda: Digenea) infection induces severe limb malformations in western toads *Bufo boreas*. Canadian Journal of Zoology **79:** 370–379.

Johnson, P.T.J., Sutherland, D.R., Kinsella, J.M., and Lunde, K.B. 2004. Review of the trematode genus *Ribeiroia* (Psilostomidae): Ecology, life history and pathogenesis with special emphasis on the amphibian malformation problem. In Advances in Parasitology, (Elsevier), pp. 191–253.

Johnson, P.T.J., and Hartson, R.B. 2009. All hosts are not equal: explaining differential patterns of malformations in an amphibian community. Journal of Animal Ecology **78**: 191–201.

Kiesecker, J.M., and Skelly, D.K. 2001. Effects of disease and pond drying on gray tree frog growth, development, and survival. Ecology **82**: 1956–1963.

Lafferty, K.D., and Morris, A.K. 1996. Altered behavior of parasitized killifish increases susceptibility to predation by bird final hosts. Ecology **77**: 1390.

Lagrue, C., Kelly, D.W., Hicks, A., and Poulin, R. 2011. Factors influencing infection patterns of trophically transmitted parasites among a fish community: host diet, host–parasite compatibility or both? Journal of Fish Biology **79**: 466–485.

Levri, E.P. 1998. The influence of non-host predators on parasite-induced behavioral changes in a freshwater snail. Oikos **81**: 531.

Moore, J. 1995. The behavior of parasitized animals. BioScience **45**: 89–96.

Mouritsen, K.N. 1997. Crawling behaviour in the bivalve *Macoma balthica*: The parasite-manipulation hypothesis revisited. Oikos **79**: 513.

Mouritsen, K.N., and Poulin, R. 2003. Parasite-induced trophic facilitation exploited by a non-host predator: a manipulator's nightmare. International Journal for Parasitology **33**: 1043–1050.

Orlofske, S.A., Belden, L.K., and Hopkins, W.A. 2009. Moderate *Echinostoma trivolvis* infection has no effects on physiology and fitness-related traits of larval pickerel frogs (*Rana palustris*). Journal of Parasitology **95**: 787–792.

Orlofske, S.A., Belden, L.K., and Hopkins, W.A. 2013. Larval wood frog (*Rana [=Lithobates] sylvatica*) development and physiology following infection with the trematode parasite, *Echinostoma trivolvis*. Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology **164**: 529–536.

Packer, C., Holt, R.D., Hudson, P.J., Lafferty, K.D., and Dobson, A.P. 2003. Keeping the herds healthy and alert: implications of predator control for infectious disease. Ecology Letters **6**: 797–802.

Poulin, R., Brodeur, J., and Moore, J. 1994. Parasite Manipulation of Host Behaviour: Should Hosts Always Lose? Oikos **70**: 479-484.

Poulin, R. 1994. Meta-analysis of parasite-induced behavioural changes. Animal Behaviour **48**: 137–146.

Poulin, R. 2010. Parasite Manipulation of Host Behavior: An Update and Frequently Asked Questions. In H. Jane Brockmann, editor: Advances in the Study of Behavior,

Burlington: Academic Press, 41: 151-186.

Schotthoefer, A.M., Koehler, A.V., Meteyer, C.U., and Cole, R.A. 2003. Influence of *Ribeiroia ondatrae* (Trematoda: Digenea) infection on limb development and survival of northern leopard frogs (*Rana pipiens*): effects of host stage and parasite-exposure level. Canadian Journal of Zoology **81**: 1144–1153.

Spindler, E. M., Zahler, M., & Loos-Frank, B. 1986. Behavioural aspects of ants as second intermediate hosts of *Dicrocoelium dendriticum*. Parasitology Research, **72**: 689-692.

Swennen, C., and Ching, H.L. 1974. Observations on the trematode *Parvatrema affinis*, causative agent of crawling tracks of *Macoma balthica*. Netherlands Journal of Sea Research **8**: 108–115.

Temple, S.A. 1987. Do predators always capture substandard individuals disproportionately from prey populations? Ecology **68**: 669-674.

4. HOST-PARASITE BEHAVIORAL INTERACTIONS BETWEEN *METAGONIMOIDES OREGONENSIS* CERCARIAE AND THE SECOND INTERMEDIATE SALAMANDER HOST, *DESMOGNATHUS QUADRAMACULATUS*

J. A. Wyderko, J. M. Wojdak, L. K. Belden

4.1 ABSTRACT

Organisms in aquatic environments frequently use chemical cues to guide interspecific interactions, especially to assess risk from natural enemies such as predators and parasites. Behavioral alteration by amphibians in response to predators has been well documented, and more recent work has shown amphibians respond similarly to free-living parasites, such as trematodes. Anti-parasite behavior may help larval amphibians avoid infection or decrease infection intensity. Conversely, parasites often use a variety of environmental cues, including chemical gradients, to orient towards potential hosts. *Metagonimoides oregonensis* is a digenetic trematode that sexually reproduces in raccoons then infect the pleurocerid snail *Elimia proxima* as first intermediate hosts, followed by encystment in the muscle tissue of the second intermediate host, frequently *Desmognathus quadramaculatus*. The goals of this study were to 1) determine whether *M. oregonensis* cercariae use chemical cues to find *D. quadramaculatus,* and to 2) determine whether *D. quadramaculatus* use behavioral mechanisms to reduce their infection level.

4.2 INTRODUCTION

Many organisms in aquatic environments rely on chemical cues to assess their environment and make decisions regarding interspecific and intraspecific interactions (Chivers and Smith 1998, Ferrari 2010). Much of the research in this area has focused on how prey may decrease

their risk of predation by altering their behavior in response to conspecific alarm cues or predator specific kairomones (Chivers and Smith 1998, Lima and Dill 1990). Behavioral changes may include area avoidance, decreased swimming and increased refuge use (Kats et al. 1988). In recent years, predators and parasites have begun to be recognized as overlapping types of natural enemies with similar effects on regulation of their resource species (Hall et al. 2008, Raffel et al. 2008). As a consequence, parasites and predators often induce similar behavioral responses from their host or prey (Werner and Peacor 2003).

Amphibians, in particular, appear to rely heavily on these types of chemical signals to avoid predation (Lutterschmidt et al. 1994, Marvin et al. 2004, Mirza et al. 2006) and will display avoidance responses in the presence of predators, most typically by increased refuge use and a decrease in activity (Kats et al. 1988). Similarly, some amphibians will decrease their activity level in the presence of parasites. Thiemann and Wassersug (2000) found that *Rana clamitans* and *R. sylvatica* tadpoles decrease their activity in the presence of echinostomatid cercariae, possibly to hinder detection by the parasite. There is also some evidence that amphibians use chemical cues to sense trematodes in the environment, and may even avoid certain areas based on parasite presence (Kiesecker and Skelly 2000, Rohr et al. 2009). Once a parasite has made physical contact, both *Rana sylvaticus* and *Bufo americanus* tadpoles will exhibit explosive swimming movements in an attempt to dislodge the parasite before penetration (Taylor et al. 2004). In this way tadpoles may be able to mediate their own infection; however, this behavior can be costly if tadpoles are simultaneously at risk from predators. In the presence of predators, tadpoles often cease defensive behaviors and so are more likely to become infected (Thiemann and Wassersug 2000). These studies indicate that the presence of parasites may elicit complex behavioral responses by amphibians, depending on the context of the interaction.

Metagonimoides oregonensis is a stream-dwelling trematode that uses raccoons, *Procyon lotor*, as definitive hosts (Price 1931, Sawyer 1958, Schaffer et al. 1961). The adult worms sexually reproduce in the small intestine, and eggs containing miracidia are shed with feces into a stream. Miracidia infect the first intermediate host, the pleurocerid snail *Elimia (=Goniobasis) proxima* (Burns and Pratt 1953). Within the snail the parasite undergoes clonal amplification, resulting in the release of cercariae. The free-living cercariae leave the snail and search for second intermediate hosts, which in the southeastern U.S. are often stream-dwelling plethodontid salamanders (Goater et al. 1987, Belden et al. 2012, Wyderko et al. 2013). *Metagonimoides oregonensis* is capable of infecting a variety of salamander species, but *Desmognathus quadramaculatus* may be a preferred host due to its long larval period and high relative abundance in streams (Wyderko et al. 2013). The cercariae penetrate the salamander and encyst as metacercariae in the muscle tissue, where they remain dormant until the salamander is consumed by the definitive host (Burns and Pratt 1953).

Some larval trematodes may also rely on chemical cues to find potential hosts in the aquatic environment. Most free-living cercariae live anywhere from a few hours to three days and are only infective for a fraction of that time (Combes 1994, Haas 2003). To maximize their odds of transmission in this short time frame, many species have fixed behavioral patterns that orient them towards habitat likely to be occupied by a potential host (Haas 1992, Haas 1994). These patterns are based on a variety of reliable environmental cues and may include responses to light, temperature, salinity, and gravity (Haas 1990, Combes 1994, Fingerut 2003, Smith 2012). The reliance on and response to these stimuli vary based on the particular trematode species and the age of the cercariae (Haas 1994, Loy 2001, Haas 2003, Koprivnikar 2010). Once they have located potential host habitat, encountering a host is thought to be largely random, especially

when potential hosts are highly motile (Combes 2002). However, some trematodes orient to their host within the habitat based on chemical gradients. Most of this work has been done in pond systems and has focused on *Echinostoma sp.* and *Schistosoma sp.* (Haas 1994a, 1994b). Use of chemical gradients is less well studied in marine and lotic systems and is highly variable among species.

The goals of these studies were to: (1) determine whether *M. oregonensis* cercariae are attracted to salamander chemical cues; (2) determine whether salamanders alter their behavior in response to the presence of chemical cues from *M. oregonensis* cercariae; and (3) determine whether these behaviors are effective at reducing the rate of infection.

4.3 MATERIALS AND METHODS

Study 1: Cercariae recognition of host chemical cue

To determine whether *M. oregonensis* cercariae use chemical cue gradients to find salamanders, we exposed free-living cercariae to three chemical cue treatments: water conditioned with *D. quadramaculatus* salamanders, *E. proxima* snails, or a non-host aquatic insect (Tipulidae larvae) .We then tested whether cercariae were attracted to the cue versus non-conditioned dechloraminated water.

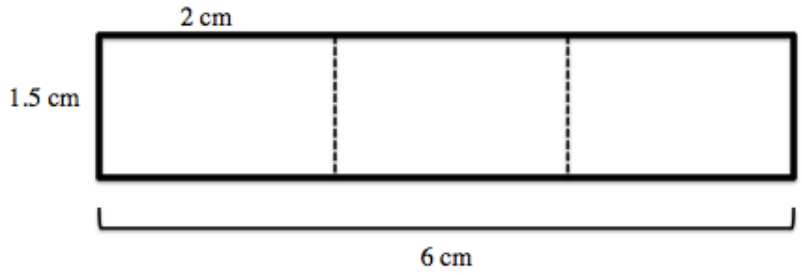
Desmognathus quadramaculatus were collected from Adam's Branch (n=2, Floyd County, VA) and Spruce Run (n=1, Giles County, VA) and were identified morphologically in the field (Petranka 1998). Tipulid larvae (n=3) were collected from Poverty Creek (n=3, Montgomery County, VA). Salamanders and tipulid larvae were housed individually in 2.3 L plastic containers (270 cm x 170 cm x 80 cm) with shallow dechloraminated water. Uninfected *E. proxima s*nails used to collect chemical cues were collected from Chisholm Creek (n=2). Snails were housed in 120 mL plastic cups and kept in an 11˚C refrigerator.

In order to collect cercariae, *Elimia proxima* snails infected as first intermediate hosts with *M. oregonensis* were collected from Rush Fork and Chisholm Creek (n=5, Floyd County, VA). They were housed in individual 120 ml plastic cups in an 11˚C refrigerator prior to use. To induce cercarial shedding, snails were brought to above room temperature (~21˚C) underneath incandescent light bulbs. Cercariae were identified morphologically under a light microscope (Burns and Pratt 1953). *Metagonimoides oregonensis* were counted and collected using a glass Pasteur pipette. All cercariae used in this experiment were less than six hours old.

To condition water with chemical cues, each individual salamander, snail or tipulid larvae was placed in an aerated o-shaped enclosure containing 50 mL of dechloraminated water for 24 hours. Animals were not fed prior to being placed in these enclosures, or while in the enclosures, to avoid chemical contamination.

An acrylic plastic linear chamber was used to measure cercariae response to chemical cues (6 cm x 1.5 cm x 1.5 cm, Figure 4.1). The I-maze was evenly marked into three sections (2 cm x1.5 cm x 1.5 cm). For each trial, 20 cercariae in one mL of dechloraminated water were pipetted into the center section. The treatment and control were pipetted into either dry side immediately after cercariae addition. They were added very slowly so that the center chamber was not disturbed and there was no premature mixing of chambers. The side containing the treatment cue was randomized for each trial to avoid any directional bias. Immediately after addition of the treatment the cercariae were allowed to move freely throughout the chamber for six minutes. At the end of the trial plastic coverslips were placed between chambers to keep the cercariae from continuing to move throughout the chamber. The number of cercariae in each section was then counted under a stereomicroscope. At the end of each trial the maze was thoroughly rinsed and wiped out to remove cercariae and chemical cues.

Figure 4.1 Diagram of linear cercarial chamber



Study 2: Salamander activity response to *M. oregonensis*

We tested whether *D. quadramaculatus* salamanders alter their activity levels in response to the presence of *M. oregonensis* cercariae. Specifically, we examined whether salamanders respond to either physical contact or chemical cues. We exposed individual salamanders to one of four treatments (N=10 trials/treatment): *M. oregonensis* physical contact, *M. oregonensis* chemical cue, physical contact by *Daphnia* (zooplankton) and a control with no stimulus. *Daphnia* were included as a control to determine whether response to physical contact by *M. oregonensis* was a result of attempts to dislodge the parasite or whether activity level changes occur in response to any light physical stimulus.

Larval *D. quadramaculatus* (N=40) were collected from Lick Run (Montgomery County, VA). There are no *E. proxima* in this stream, so salamanders were assumed to be uninfected with *M. oregonensis.* Salamanders were identified morphologically in the field (Petranka 1998) and were collected at least 48 hours prior to the experiment. Salamanders were housed in an 18˚C cold room on a 12:12 light:dark cycle in individual 2.25 L Tupperware containers with shallow dechloraminated water and a large rock for cover. They were not fed prior to the experiment.

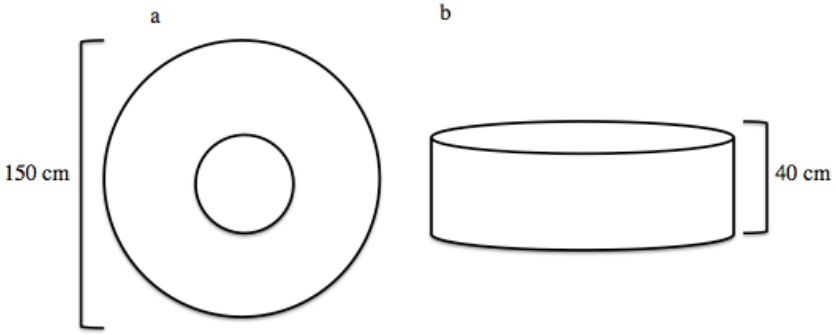
Elimia proxima snails infected with *M. oregonensis* were collected from Chisholm Creek (n=13, Floyd County, VA) and housed in individual 120 ml plastic cups in an 11˚C refrigerator prior to use. Cercariae for trials were collected as described above. All cercariae used in this experiment were less than six hours old. *Daphnia* were collected from populations established in mesocosms stock tanks at Virginia Tech's Kentland Farm (Montgomery County, VA) and were counted out under a stereomicroscope.

For each of the four treatments, a cage constructed using steel wire and nitex mesh (1.27 cm x 2.54 cm x 2.54 cm) was used to contain the treatment. The size of the nitex mesh varied depending on whether the treatment was testing response to physical contact (100 μm) or response to chemical cue (30 μm). To test response to physical contact by cercariae we exposed individuals in this group to 100 cercariae using 100 μm mesh, which allowed the cercariae to disperse from the cage and make physical contact with the salamanders. To test response to cercariae chemical cue we exposed individuals in this group to 100 cercariae using 30 μm mesh, which kept cercariae from making physical contact with the salamanders, but allowed for dispersal of chemical cues. To determine whether response to physical contact was parasite-specific we exposed individuals in this group to 100 microscopic *Daphnia* housed in 100 μm. The last treatment group was exposure to one mL dechloraminated water, which acted as a blank control stimulus.

Experimental enclosures were donut shaped (150 cm diameter x 40 cm height) with circular flow maintained by an aquarium pump. Enclosures were filled with 200 mL dechloraminated water and the proper cage according to treatment was placed inside, directly across from the air pump. Salamanders were removed from individual housing using a mesh aquarium net and placed in the experimental enclosure. The treatment stimulus was then added

to the cage using a glass Pasteur pipette. Three trials were set-up at one time. Salamanders were allowed to acclimate for one minute. Activity was then recorded for an additional 15 minutes using a Sony video camera (HDR-XR500V or DCR-SR68). Post-experiment, salamanders were returned to their individual housing containers. Enclosure water was changed in between each trial. Total time active (out of 15 minutes) was assessed from the video recordings.

Figure 4.2 Diagram of salamander experimental enclosures (a) view from top looking down, (b) side view.



Salamanders were housed individually in the lab for four weeks post experiment to allow time for any potential infections to mature. They were fed three blackworms every three days. During this time one salamander escaped from its enclosure and died. All other salamanders survived. After four weeks, salamanders were euthanized using an overdose of buffered 0.5% Tricaine methanesulfonate (MS-222). After euthanasia, salamanders were transferred to formalin for 24 hours, followed by a formalin rinse for an additional 24 hours, and finally transferred to 70% ethanol for storage. Mass and snout-vent length was recorded for all salamanders. To look for the presence of *M. oregonensis* metacercariae salamanders underwent a modified version of Hanken and Wassersug's (1981) clearing and staining protocol (as described in Wyderko et al. 2013). Following clearing and staining, salamanders were stored in 100% glycerin with a thymol crystal until total cyst counts were completed.

Study 3: Effects of salamander behavioral response on infection level

The goal of this study was to determine whether *D. quadramaculatus* are capable of reducing infection through behavioral mechanisms. We anaesthetized one group of salamanders prior to exposure and compared metacercariae infection levels to the control group, which were awake and allowed to behave normally (as in Koprivnikar et al. 2006).

Larval *D. quadramaculatus* (0.36 g ±0.11) salamanders were collected from Lick Run (n=20, Montgomery County, Virginia) at least seven days prior to the experiment. Salamanders were not fed and were housed as described above. Behavioral experiments were performed in an 18˚C room during the dark cycle under red light because these salamanders are nocturnal. The trials were performed over the course of two nights, each time with ten salamanders.

Elimia proxima snails infected with *M. oregonensis* were collected from Howell Creek (n=10). They were housed and cercariae were collected as described above. Cercariae used in the experiment were never more than three hours old.

Salamanders were randomly assigned to either the “awake” or “anaesthetized” group. In order to anaesthetize salamanders, they were placed in individual 50 mL centrifuge tubes containing 15 mL of buffered 0.1% MS-222 for 15 minutes. Salamanders in the "awake" group underwent the same procedure, however instead of buffered MS-222, tubes had 15 mL of dechloraminated water. Each individual was then rinsed with dechloraminated water and placed in individual aerated donut shaped container, as described above. Cercariae (n=100) were pipetted into each enclosure and salamanders were exposed for 30 minutes. After the 30 minute exposure, salamanders were rinsed again and returned to their original housing containers. Salamanders were maintained in the lab for four weeks post-exposure to allow time for metacercariae to develop. Three salamanders died in their enclosures during near the end of the four week period (Two from the anaesthetized group and one from the control group). After four

weeks, salamanders were euthanized by an overdose of buffered 0.5% MS-222 and were cleared and stained as described above.

Statistical Analyses

Study 1: Cercariae recognition of host chemical cue

Cercariae distribution in the stimulus chamber fit a normal distribution across all three treatment groups. We used a generalized linear model assuming a binomial distribution to compare whether the proportion of cercariae in the stimulus chamber was significantly different among the three treatment conditions.

Study 2: Salamander activity response to *M. oregonensis*

Salamander time active was log-transformed to approximate a normal distribution. We then performed an ANOVA to test whether salamanders altered the amount of time they spent swimming in response to direct contact by cercariae or cercariae chemical cues.

Study 3: Effects of salamander behavior response on infection level

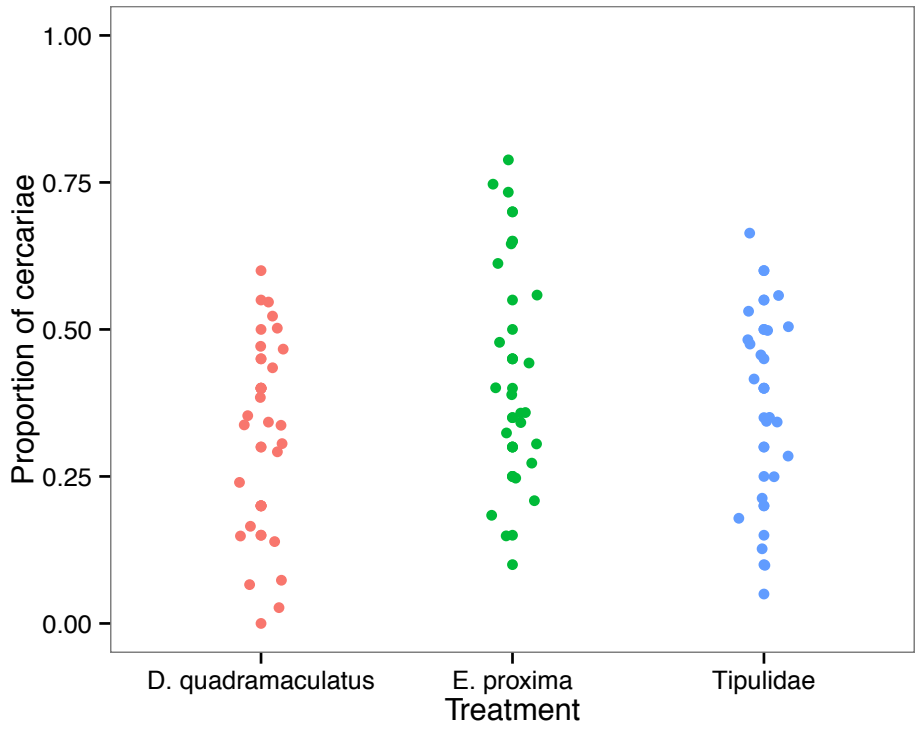
No analysis was performed on this data set because none of the individuals were infected with *M. oregonensis*.

4.4 RESULTS

Study 1: Cercariae recognition of host chemical cue

There were no significant differences among the three treatment groups in the proportion of cercariae within stimulus chamber. The proportion of cercariae in the salamander-conditioned water was not significantly different than in the snail ($p=0.588$, 0.541) or tipulid ($p=0.802$, $z=0.250$) conditioned water (Figure 4.3).

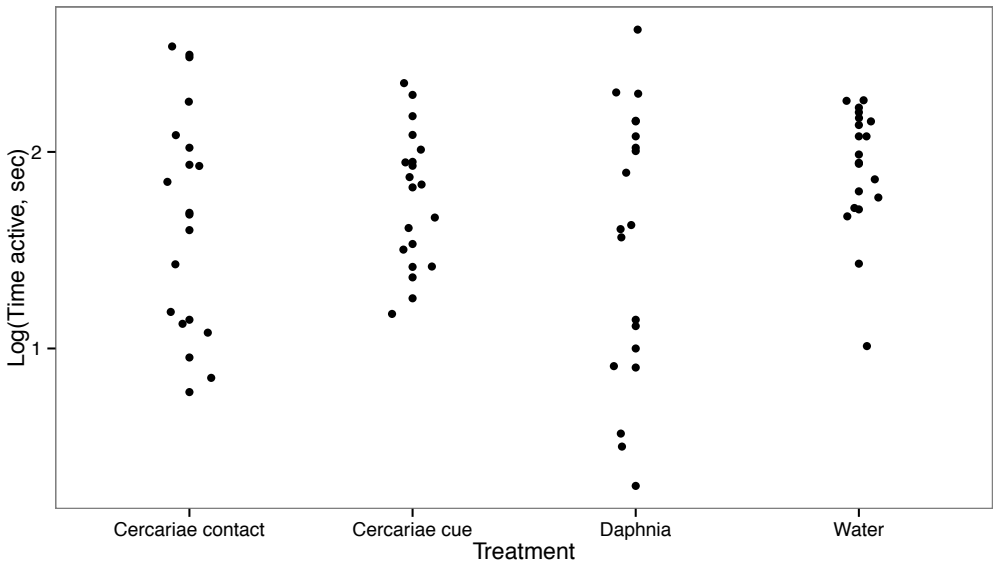
Figure 4.3 Proportion of cercariae in the stimulus chamber among the three treatment groups: salamander conditioned water, snail conditioned water, and tipulid conditioned water.



Study 2: Salamander activity response to *M. oregonensis*

Desmognathus quadramaculatus exhibited similar activity levels (average time active= 1 min 25 sec) in response to either physical contact by cercariae, the presence of cercariae chemical cues, potential physical contact by *Daphnia,* or the control unconditioned dechloraminated water (p=0.236, F=1.48, Figure 4.4).

Figure 4.4 Comparison of log of time active across four treatment groups: cercariae physical contact, cercariae chemical cue, backswimmer physical contact, and dechloraminated water.



Study 3: Effects of salamander behavior response on infection level

After clearing and staining no visible *M. oregonensis* metacercariae were found in *D. quadramaculatus.* Since there was no quantifiable infection we were unable to compare treatment groups or draw any conclusions from this experiment.

4.5 DISCUSSION

Study 1: Cercariae recognition of host chemical cue

Metagonimoides oregonensis cercariae do not appear to chemo-orient towards *D. quadramaculatus* chemical cues. These results are consistent with observations made by Burns and Pratt (1953) about the lack of cercarial response to anuran tissue. They observed that cercariae would often swim or rest within one millimeter of frog tissue without reacting, and appeared to only respond to tissue once direct physical contact had been made. In other

experimental exposures of live *D. quadramaculatus* salamanders, we have subsequently made similar observations. *Metagonimoides oregonensis* appear to make contact with salamanders largely by chance and would often swim very close to the host without apparent recognition. Direct contact with host stimulated active penetration behavior, but cercariae did not appear to actively orient and make directed movements toward hosts. We also exposed cercariae to agar gel conditioned with *D. quadramaculatus* cue (as in MacInnis 1965) to observe their penetration response. Cercariae never fully penetrated the agar. Once they made contact they appeared to attach with their anterior end and whirled the posterior end until their tail dislodged. After that point they generally continued to make a creeping motion, but without any movement around or into the agar. After about two hours they ceased movement entirely.

This lack of response to chemical cues makes sense within the context of a stream because the chemical gradient is probably quickly disrupted in fast-flowing streams, and at best can only indicate hosts upstream. It is unlikely cercariae are able to swim upstream into flow, which they would have to do to follow the gradient to the host. In a low flow gradient (0.2 cm/s) marine cercariae, *Himasthla rhigedana***,** actively swam downwards to reach the benthic zone faster than through passive sinking alone. However, when flow gradient was high (0.8 cm/s) cercariae were unable to actively orient and were instead passively and evenly distributed throughout the water column (Fingerut et al. 2003). Appalachian headwater streams are typically fast flowing environments, so *M. oregonensis* cercariae may primarily rely on environmental cues to find habitat where random host encounter is most likely, rather than chemical orientation towards a specific host (Haas 1992, Loy 2001).

Study 2: Salamander activity response to *M. oregonensis*

Desmognathus quadramaculatus did not respond to the presence of cercariae or cercariae chemical cues with a change in time spent swimming. There are several possible explanations for this lack of response. Salamanders may not respond to cercariae if the costs of parasite infection are low compared to the costs of a change in behavior. In trematode systems where larval amphibian anti-parasite behavior has been demonstrated, primarily for the trematodes *Echinostoma* sp. and *Riberoia ondatrae*, the fitness costs of metacercariae infection may be high when infection levels are high (Koprivnikar et al. 2006, Daly and Johnson 2010). In contrast *M. oregonensis* metacercariae infection appears to result in little pathology to salamander hosts, even in animals with high metacercariae burdens (Belden et al. 2012). The cost of increased swimming may be higher than the costs of infection, especially since a relatively low number of cercariae were used in this experiment. There may be a threshold level of cercariae in an environment to which a salamander will respond.

In addition, cercariae chemical cues may have been too dilute to warrant a response – some species only respond to chemical cues above certain concentrations because concentration tends to correspond with threat level (Ferrari et al 2010). Avoidance responses to parasites may also be a learned response rather than an innate response. Fathead minnows show no response to *Ornithodiplosomum* cercariae during their first exposure, but respond to chemical and visual cues with avoidance displays during subsequent exposures (James et al. 2008). The salamanders used in these experiments were naïve to *M. oregonensis* and may not have developed a learned response to the parasite.

Study 3: Effects of salamander behavior response on infection level

Since no infection was found in *D. quadramaculatus* after exposure, the conclusions we can draw from this study are limited. There are a variety of possible reasons why salamanders

did not have metacercariae. One possibility is that the exposure time (15 minutes) was too short and cercariae did not have enough time to encounter and penetrate the host. This is especially likely given our previous findings that *M. oregonensis* cercariae do not appear to chemo-orient towards *D. quadramaculatus*. We observed that the cercariae seem to be relatively easily dislodged by movement of the salamander during early contact stages, so cercariae may have been dislodged when salamanders were moved from enclosures. In addition, *D. quadramaculatus* used in this experiment were euthanized 26 days post-exposure. Burns and Pratt (1953) found that metacercariae took 45 days to mature and so salamanders may have been euthanized prematurely. If this is the case metacercariae may not have been visible after clearing and staining due to their immature state.

Overall, these studies seem to indicate that interactions between *M. oregonensis* and *D. quadramaculatus* are passive and rely largely on random encounters. Cercariae do not appear to be attracted to salamander chemical cues, suggesting that other environmental cues may be more useful for host-finding in this system. In addition, salamanders do not seem to display strong anti-parasite behavior when confronted with direct contact with cercariae or exposure to chemical cues Infection of *D. quadramaculatus* with *M. oregonensis* may not have major fitness consequences for the salamander, and so behavioral responses to the parasite have not developed.

4.6 REFERENCES

Belden, L.K., Peterman, W.E., Smith, S.A., Brooks, L.R., Benfield, E.F., Black, W.P., Yang, Z., and Wojdak, J.M. 2012. *Metagonimoides oregonensis* (Heterophyidae: Digenea) infection in pleurocerid snails and *Desmognathus quadramaculatus* salamander larvae in southern Appalachian streams. Journal of Parasitology **98**: 760–767.

Burns, W.C., and Pratt, I. 1953. The Life Cycle of *Metagonimoides oregonensis* Price (Trematoda: Heterophyidae). The Journal of Parasitology **39**: 60-69.

Chivers, D.P., and Smith, R. J. F. 1998. Chemical alarm signaling in aquatic predator-prey systems: A review and prospectus. Ecoscience **5**: 338–352.

Combes, C., Fournier, A., Mone, H., and Theron, A. 1994. Behaviors in trematode cerceriae that enhance parasite transmission - patterns and processes. Parasitology **109**: S3–S13.

Daly, E.W., and Johnson, P.T. 2010. Beyond immunity: quantifying the effects of host anti-parasite behavior on parasite transmission. Oecologia **165**: 1043–1050.

Ferrari, M.C.O., Wisenden, B.D., and Chivers, D.P. 2010. Chemical ecology of predator–prey interactions in aquatic ecosystems: a review and prospectus. Canadian Journal of Zoology **88**: 698–724.

Fingerut, J.T., Zimmer, C.A., and Zimmer, R.K. 2003. Larval swimming overpowers turbulent mixing and facilitates transmission of a marine parasite. Ecology **84**: 2502–2515.

Goater, T.M., Esch, G.W., and Bush, A.O. 1987. Helminth parasites of sympatric salamanders: Ecological concepts at infracommunity, component and compound community Levels. American Midland Naturalist **118**: 289-300.

Haas, W., Granzer, M., and Brockelman, C. 1990. *Opisthorchis viverrini*: Finding and recognition of the fish host by the cercariae. Experimental Parasitology **71**: 422–431.

Haas, W., Granzer, M., and Brockelman, C.R. 1990. Finding and recognition of the bovine host by the cercariae of *Schistosoma spindale*. Parasitology Research **76**: 343–350.

Haas, W. 1992. Physiological analysis of cercarial behavior. The Journal of Parasitology **78**: 243-255.

Haas, W., Haberl, B., Schmalfuss, G., and Khayyal, M.T. 1994a. *Schistosoma haematobium* cercarial host-finding and host-recognition differs from that of *S. mansoni*. The Journal of Parasitology **80**: 345-353.

Haas, W. 1994b. Physiological analyses of host finding behavior in trematode cercariae: adaptations for transmission success. Parasitology **109**: S15–S29.

Haas, W. 2003. Parasitic worms: strategies of host finding, recognition and invasion. Zoology **106**: 349–364.

Hall S.R., Lafferty K.D., Brown J.M., Caceres C.E., Chase J.M., Dobson A.P., Holt R.D., Jones C.G., Randolph S.E., Rohani P. 2008. Is infectious disease just another type of predator-prey interaction? In: Ostfeld R.S., Keesing F., Eviner V.T. (eds) Infectious disease ecology: effects of ecosystems on disease and of disease on ecosystems.

Princeton University Press, New Jersey

James, C.T., Noyes, K.J., Stumbo, A.D., Wisenden, B.D., and Goater, C.P. 2008. Cost of exposure to trematode cercariae and learned recognition and avoidance of parasitism risk by fathead minnows (*Pimephales promelas*). Journal of Fish Biology **73**: 2238–2248.

Kats, L.B., Petranka, J.W., and Sih, A. 1988. Antipredator defenses and the persistence of amphibian larvae with fishes. Ecology **69**: 1865-1870.

Kiesecker, J.M., and Skelly, D.K. 2000. Choice of oviposition site by gray treefrogs: The role of potential parasitic infection. Ecology **81**: 2939–2943.

Koprivnikar, J., Forbes, M.R., and Baker, R.L. 2006. On the efficacy of anti-parasite behaviour: A case study of tadpole susceptibility to cercariae of Echinostoma trivolvis. Canadian Journal of Zoology **84**: 1623–1629.

Koprivnikar, J., Lim, D., Fu, C., and Brack, S.H.M. 2010. Effects of temperature, salinity, and pH on the survival and activity of marine cercariae. Parasitology Research **106**: 1167–1177.

Lima, S.L., and Dill, L.M. 1990. Behavioral decisions made under the risk of predation: a review and prospectus. Canadian Journal of Zoology **68**: 619–640.

Loy, C., Motzel, W., and Haas, W. 2001. Photo- and geo-orientation by Echinostome cercariae results in habitat selection. Journal of Parasitology **87**: 505–509.

Lutterschmidt, W.I., Marvin, G.A., and Hutchison, V.H. 1994. Alarm response by a plethodontid salamander (*Desmognathus ochrophaeus*): Conspecific and heterospecific "Schreckstoff". Journal of Chemical Ecology **20**: 2751–2759.

MacInnis, A.J. 1965. Responses of Schistosoma mansoni Miracidia to Chemical Attractants. The Journal of Parasitology **51**: 731–746.

Marvin, G.A., Whitekiller, R.R., and Hutchison, V.H. 2004. Avoidance of alarm chemicals by plethodontid salamanders (Genus *Eurycea*): Importance of phylogeny, ecology, and methodology. Herpetologica **60**: 24-33.

Mirza, R., Ferrari, M., Chivers, D., and Kiesecker, J. 2006. Responses of American toad tadpoles to predation cues: Behavioural response thresholds, threat-sensitivity and acquired predation recognition. Behaviour **143**: 877–889.

Petranka, J. 1998. Salamanders of the United States and Canada. Washington: Smithsonian Institution Press.

Price, E. W. 1931. *Metagonimoides oregonensis*, a new trematode from a raccoon. Journal of the Washington Academy of Sciences **21**: 405-407.

Rohr, J.R., Swan, A., Raffel, T.R., and Hudson, P.J. 2008. Parasites, info-disruption, and the ecology of fear. Oecologia **159**: 447–454.

Sawyer, T. K. 1958. *Metagonimoides oregonensis* Price, 1931 from a Georgia Raccoon with a Note on *Sellacotyle mustelae* Wallace, 1935. The Journal of Parasitology **44**: 63.

Schaffer, G. D., W. R. Davidso, V. F. Nettles, E. A. Rollor. 1961. Helminth parasites of translocated raccoons (*Procyon lotor*) in the southeastern United States. Journal of Wildlife Diseases **17**: 217-227.

Schell, S. C. 1985. Trematodes of North America. University of Idaho Press, Moscow, Idaho: 263 pages.

Smith, N.F., and Cohen, J.H. 2012. Comparative photobehavior of marine cercariae with differing secondary host preferences. Biol Bull **222**: 74–83.

Taylor, C.N., Oseen, K.L., and Wassersug, R.J. 2004. On the behavioural response of *Rana* and *Bufo* tadpoles to echinostomatoid cercariae: implications to synergistic factors influencing trematode infections in anurans. Canadian Journal of Zoology **82**: 701–706.

Thiemann, G.W., and Wassersug, R.J. 2000. Patterns and consequences of behavioural responses to predators and parasites in *Rana* tadpoles. Biological Journal of the Linnean Society **71**: 513–528.

Werner, E.E., and Peacor, S.D. 2003. A review of trait-mediated indirect interactions in ecological communities. Ecology **84**: 1083–1100.

Wyderko, J.A., E. F. Benfield, J.C. Maerz, K.C. Cecala, L.K. Belden. 2013. Variable infection of stream salamanders in the southern Appalachains by the trematode *Metagonimoides oregonensis* (Price 1931, Family: Heterophyidae). *In review*.

5. CONCLUSIONS

Metagonimoides oregonensis appears capable of infecting multiple plethodontid salamander species as second intermediate hosts. However, patterns of infection prevalence and intensity vary among these hosts. *Desmognathus quadramaculatus* appear to have very high prevalence and intensity of infection so they may be more important in the transmission cycle of this parasite. One factor that may explain this distribution of infection is the varying length of the larval period of the different salamander species. *Desmognathus quadramaculatus* have a comparatively long larval period lasting three to four years, which probably results in greater exposure to *M. oregonensis*. Interestingly the only other species with similarly high infection intensity, *Gyrinophilus porphyriticus*, also has a fairly long larval period, although due to the small sample size we weren't able to include *G. porphyriticus* in our statistical analysis. Larval period is probably just one of several ecological and environmental factors influencing *M. oregonensis* distributions.

Relatively general use of second intermediate hosts probably plays an important role in transmission dynamics of the parasite. Raccoons tend to consume anything they can easily find or catch. Since they are not specifically predating salamanders, odds of transmission may be increased with a greater number of potential hosts. Even with multiple potential second intermediate hosts *D. quadramaculatus* may play a larger role in transmission due to their high infection levels and high relative abundances in streams.

In the second part of this study the role of both parasite and salamander host behavior in infection dynamics was explored. Host-parasite interaction between *M. oregonensis* cercariae and *D. quadramaculatus* seem to be driven largely by chance encounters. Cercariae do not appear to orient towards the host via chemical cues. Since host chemical gradients are probably

unreliable in fast-flowing streams, it seems more likely that cercariae rely primarily on environmental cues to localize in host space and time (Combes et al. 1994). Another species in this trematode family uses photo-taxis (Smith et al. 2012), and the presence of large eye spots indicate light may be an important cue for *M. oregonensis* as well. Snails release huge numbers of clonal cercariae into the environment and as long as some portion of these cercariae succeed in finding a host, the parasite will be maintained in the system. As a result, there may be no need for cercariae to develop highly specialized host-finding mechanisms if the chances of encounter of a host in a certain type of habitat are high (Combes et al. 2002). Salamanders are frequently found in dark areas under rocks, especially during the daytime. It is possible cercariae are negatively photo-tactic and localize toward this type of habitat. Underneath rocks and at the benthic layer there is little effect of flow, so cercariae may be more likely to find salamanders in these confined, low-flow microhabitats.

Desmognathus quadramaculatus do not appear to react behaviorally to the presence of cercariae or cercariae chemical cue in the environment. Lack of response by the salamander may make sense if infection is non-pathogenic and the cost of a behavioral reaction is greater than the cost of infection. I did not find any effect of metacercariae infection on salamander locomotor performance with moderate levels of infection, indicating moderate infections may be relatively non-pathogenic. Furthermore, Belden et al. (2012) found no histo-pathology associated with *M. oregonensis* metacercariae infection in larval salamanders, so it seems possible that a behavioral response would be an unnecessary expense of energy. If metacercariae decreased locomotor performance, salamanders could potentially be more susceptible to capture by the raccoon definitive host; however, it is likely that any locomotor effects that increased susceptibility to predation by a raccoon would also increase susceptibility to predation by a dead-end host (i.e.

snakes, opossums). Since even very young larval salamanders are capable of carrying huge metacercariae burdens, raccoons may become infected with *Metagonimoides oregonensis* very easily.

Metagonimoides oregonensis may also have other effects on salamanders not measured in this study. Overall, the lack of behavioral interactions indicate that behavior may not be an important factor in explaining *Metagonimoides oregonensis* distribution among second intermediate hosts. Future work examining parasite distributions and transmission in this system should consider a number of other environmental and ecological factors that affect parasite distribution including spatial heterogeneity in snail distribution, salamander body sizes, or effects of temporal breeding patterns.

REFERENCES

Belden, L.K., and Wojdak, J.M. 2011. The combined influence of trematode parasites and predatory salamanders on wood frog (*Rana sylvatica*) tadpoles. Oecologia **166**: 1077–1086.

Burns, W.C., and Pratt, I. 1953. The life cycle of *Metagonimoides oregonensis* Price (Trematoda: Heterophyidae). The Journal of Parasitology **39**: 60-69.

Combes, C., Fournier, A., Mone, H., and Theron, A. (1994). Behaviors in trematode cerceriae that enhance parasite transmission - patterns and processes. Parasitology *109*, S3–S13.

Combes, C., Bartoli, P., and Theron, A. 2002. Trematode transmission strategies. In The Behavioral Ecology of Parasites, CABI Publishing, pp. 1–12.

Daly, E.W., and Johnson, P.T.J. 2010. Beyond immunity: quantifying the effects of host anti-parasite behavior on parasite transmission. Oecologia **165**: 1043–1050.

Detwiler, J.T., and Minchella, D.J. 2009. Intermediate host availability masks the strength of experimentally-derived colonisation patterns in echinostome trematodes. International Journal for Parasitology **39**: 585–590.

Evans, N.A., and Gordon, D.M. 1983. Experimental observations on the specificity of *Echinoparyphium recurvatum* toward second intermediate hosts. Parasitology Research **69**: 217–222.

Hudson, P., Dobson, A., and Newborn, D. 1992. Do Parasites Make Prey Vulnerable to Predation - Red Grouse and Parasites. J. Anim. Ecol. **61**: 681–692.

Hudson, P.J., Dobson, A.P., and Lafferty, K.D. 2006. Is a healthy ecosystem one that is rich in parasites? Trends in Ecology & Evolution **21**: 381–385.

Kiesecker, J.M., and Skelly, D.K. 2000. Choice of oviposition site by gray treefrogs: The role of potential parasitic infection. Ecology **81**: 2939–2943.

Koprivnikar, J., Forbes, M.R., and Baker, R.L. 2008. Larval amphibian growth and development under varying density: are parasitized individuals poor competitors? Oecologia **155**: 641–649.

Kuris, A.M., Hechinger, R.F., Shaw, J.C., Whitney, K.L., Aguirre-Macedo, L., Boch, C.A., Dobson, A.P., Dunham, E.J., Fredensborg, B.L., Huspeni, T.C., et al. 2008. Ecosystem energetic implications of parasite and free-living biomass in three estuaries. Nature **454**: 515–518.

Lafferty, K.D., Allesina, S., Arim, M., Briggs, C.J., De Leo, G., Dobson, A.P., Dunne, J.A., Johnson, P.T.J., Kuris, A.M., Marcogliese, D.J., et al. 2008. Parasites in food webs: the ultimate missing links. Ecology Letters **11**: 533–546.

Lewis, E. E., J. F. Campbell, et al. 2002. The Behavioural Ecology of Parasites. New York, CABI Publishing.

Lutterschmidt, W.I., Marvin, G.A., and Hutchison, V.H. 1994. Alarm response by a plethodontid salamander *Desmognathus ochrophaeus*: Conspecific and heterospecific "Schreckstoff". Journal of Chemical Ecology **20**: 2751–2759.

Marcogliese, D.J. 2004. Parasites: Small Players with Crucial Roles in the Ecological Theater. EcoHealth **1**: 151–164.

Marcogliese, D.J., and Cone, D.K. 1997. Food webs: a plea for parasites. Trends in Ecology & Evolution **12**: 320–325.

Marvin, G.A., Whitekiller, R.R., and Hutchison, V.H. 2004. Avoidance of alarm chemicals by plethodontid salamanders (genus *Eurycea*): Importance of phylogeny, ecology, and methodology. Herpetologica **60**: 24–33.

Minchella, D.J., and Scott, M.E. 1991. Parasitism: A cryptic determinant of animal community structure. Trends in Ecology & Evolution **6**: 250–254.

Mirza, R., Ferrari, M., Chivers, D., and Kiesecker, J. 2006. Responses of American toad tadpoles to predation cues: behavioural response thresholds, threat-sensitivity and acquired predation recognition. Behaviour **143**: 877–889.

Mouritsen, K.N., and Poulin, R. 2005. Parasites boosts biodiversity and changes animal community structure by trait-mediated indirect effects. Oikos **108**: 344–350.

Raffel, T.R., Hoverman, J.T., Halstead, N.T., Michel, P.J., and Rohr, J.R. 2010. Parasitism in a community context: trait-mediated interactions with competition and predation. Ecology **91**: 1900–1907.

Rohr, J.R., Swan, A., Raffel, T.R., and Hudson, P.J. 2008. Parasites, info-disruption, and the ecology of fear. Oecologia **159**: 447–454.

Smith, N.F., and Cohen, J.H. (2012). Comparative photobehavior of marine cercariae with differing secondary host preferences. Biol Bull **222**: 74–83.

Spindler, E. M., M. Zahler, et al. (1986). "Behavioral aspects of ants as second intermediate hosts of Dicrocoelium dendriticum." Z Parasitenkd **72**: 689-692.

Taylor, C.N., Oseen, K.L., and Wassersug, R.J. (2004). On the behavioural response of *Rana* and *Bufo* tadpoles to echinostomatoid cercariae: implications to synergistic factors influencing trematode infections in anurans. Canadian Journal of Zoology **82**: 701–706.

Thiemann, G.W., and Wassersug, R.J. 2000. Patterns and consequences of behavioural responses to predators and parasites in Rana tadpoles. Biological Journal of the Linnean Society **71**: 513–528.

Thomas, F., Poulin, R., De Meeüs, T., Guégan, J.-F., Renaud, F., Thomas, F., De Meeus, T., Guegan, J.-F., and Renaud, F. 1999. Parasites and Ecosystem Engineering: What Roles Could They Play? Oikos **84**: 167.

Turner, A.M. 2008. Predator diet and prey behaviour: freshwater snails discriminate among closely related prey in a predator's diet. Animal Behaviour **76**: 1211–1217.

Wood, C.L., Byers, J.E., Cottingham, K.L., Altman, I., Donahue, M.J., and Blakeslee, A.M.H. 2007. Parasites alter community structure. Proceedings of the National Academy of Sciences **104**: 9335–9339.