Article

Effects of vertical position on trematode parasitism in larval anurans

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Abstract

Spatial distributions of animals can affect interactions with their natural enemies, such as parasites, and thus have important implications for host–parasite dynamics. While spatial variation in infection risk has been explored in many systems at the landscape scale, less attention has been paid to spatial structure at smaller scales. Here, we explore a hypothesized relationship between a common spatial variable, vertical position, and risk of parasite infection in a model aquatic system, larval frogs (Rana) and trematode (Digenea) parasites. Vertical position is relevant to this system given evidence that the densities of snail first intermediate hosts, tadpole second intermediate hosts, and trematode infective stages can vary with depth. To test the effects of depth on infection risk of larval frogs by trematodes, we performed two enclosure experiments, one in the laboratory and one in the field, in which larval frogs in cages just below the water surface or near the bottom of the water column were exposed to parasites. Compared with near-surface cages, mean infection load (number of cysts) in tadpoles in near-bottom cages was 83% higher after 48-h exposures in the laboratory and 730% higher after 10-day exposures in the field. Our findings thus indicate that infection risk depends on depth, which may have adaptive significance, as tadpoles have previously been shown to change vertical position in response to parasite presence. These results motivate future work examining vertical variation in infection risk and may have broader implications for host–parasite dynamics and evolution of host and parasite behavior.

Key words: avoidance behavior, cercariae, Echinostomatidae, host–parasite interactions, Rana, spatial structure
Behavior plays a critical role in determining animal spatial position and susceptibility to natural enemies. For instance, prey may favor open versus structured habitats based on perceived risk depending on predator abundance and composition (Meutter et al. 2005). Similarly, host behavior can influence parasitism (e.g., contact and infection rates) across a variety of host–parasite systems via several mechanisms, such as spatial and temporal avoidance, parasite removal, and post-infection behaviors to reduce detrimental effects (e.g., Moore and Freehling 2002; Milan et al. 2012; Bui et al. 2019). In particular, avoidance behavior is the first line of defense for hosts, due to typically lower resource costs than immunity (Zuk and Stoehr 2002), and in some instances can be more important for mitigating infection risk than immunological defenses (e.g., Daly and Johnson 2011). Spatial avoidance behaviors, such as vertical movement in the water column by aquatic animals, may thus be a relatively low-cost strategy to reduce infection risk.

Here, we assess whether water column position affects the risk of infection in a system where a vertical behavioral response to parasites has previously been observed. Specifically, we examined how infection in Rana (=Lithobates) tadpoles by a common group of trematode parasites, echinostomes (Digenea: Echinostomatidae), depends on host vertical position. A previous study found that the proportion of Rana clamitans tadpoles (common second intermediate hosts) at the water surface is 253% higher when infective stages (cercariae) of echinostomes are present (Marino 2016a). We hypothesize that this vertical movement behavior by the hosts is an adaptive response to vertical variation in infection risk. Such variation in infection risk could arise because, for example, echinostome cercariae orient vertically using light cues from the water surface (McCarthy 1999; Platt and Dowd 2012). For instance, cercariae of several echinostome species have been observed in the laboratory to aggregate toward the bottom of the water column (Loy et al. 2001), which could cause increased infection risk with increased depth. Furthermore, the density of snail first intermediate hosts from which the cercariae emerge could also be higher near the bottom (Boag 1981), which could also increase infection risk in deeper water. However, other factors may counteract the effects of downward cercariae movement or vertical structure in snail distributions on infection risk. For instance, predation on cercariae by benthic invertebrates (Orlofske et al. 2012; Rohr et al. 2015) may reduce cercariae abundance near the bottom of the water column. We thus performed experiments to test for the presence of vertical variation in infection risk in both the laboratory and field. In both settings, we used enclosures to restrict tadpole vertical position while exposed to trematode cercariae. We then quantified infection and assessed whether observed spatial variation was consistent with our hypothesis that the previously observed vertical movement in response to parasite presence is adaptive.

Materials and Methods

Study system

Echinostomatidae is a widely distributed family of digenetic trematodes and a useful model for understanding host–parasite interactions (Johnson and McKenzie 2009). The echinostome life cycle involves three hosts (reviewed by Szuroczki and Richardson 2009). Adult echinostomes reproduce sexually in the intestinal tract of a bird or mammal definitive host, and eggs are passed through the host’s feces. Eggs hatch into miracidia, which then infect an aquatic snail first intermediate host. After entering the snail, asexual reproduction occurs, after which free-swimming cercariae emerge and infect the second intermediate host (commonly larval amphibians or another snail); the echinostome life cycle is complete when the definitive host consumes the second intermediate host and the parasite matures to the adult stage.

In tadpoles, echinostome cercariae infect the nephric system (mesonephri, nephric ducts, and pronephri) by entering through the cloaca (Thiennemann and Wassersug 2000). Infection of Rana tadpoles can cause edema (Schorthoef er et al. 2003), age- and stage-dependent mortality (Holland et al. 2007), and delayed onset of metamorphosis (Orlofske et al. 2017). A variety of behaviors in larval amphibians have been linked to reduced risk of infection by trematodes. For species such as R. clamitans and R. catesbeiana, a generalized increase in activity is linked to reduced risk of infection by echinostome cercariae (Koprivnikar et al. 2006; Koprivnikar et al. 2014). Rana and Bufo tadpoles also utilize sudden bursts of speed and twisting when they sense cercariae on their skin, a behavior that helps to dislodge parasites and thereby reduce infection (Taylor et al. 2004). Finally, tadpoles have also been shown to engage in a horizontal avoidance response to the presence of cercariae (Rohr et al. 2009).

Animal collection and maintenance

All amphibians used in experiments were collected as egg masses from wetlands near Peoria, IL during June 2017. As these wetlands are located on privately owned land, permission was obtained from landowners prior to retrieving eggs. We collected two partial R. catesbeiana egg masses from two ponds (used in the lab experiment) and two partial R. clamitans egg masses from a single pond (used in the field experiment). Different species were used in the lab and field experiments due to availability of tadpoles at the initiation of each experiment; because these species are closely related (Hills and Wilcox 2006) and similar in their ecology (Herrick et al. 2018), we expected that both species would respond similarly to exposure to echinostome cercariae (Koprivnikar et al. 2006, 2014; Marino 2016b). Eggs were reared to tadpoles in 19 L aquaria containing reverse osmosis-filtered water at 21°C under 12 h:12 h light:dark cycle in the Animal Care Facility at Bradley University. After hatching, tadpoles were fed Great Choice® Rabbit Food ad libitum prior to use in the experiment. All research was performed in accordance with guidelines from Bradley University’s Institutional Animal Care and Use Committee.

Laboratory experiment

To examine vertical structure in infection risk in a controlled setting, we conducted a laboratory experiment using tadpoles in enclosures...
To examine vertical structure in infection risk in a field setting, we conducted an experiment to measure parasite infection in tadpoles placed in enclosures either at the surface or near the bottom of a pond. Based on a field survey of trematodes in ponds in central Illinois prior to the experiment (Steenrod et al., unpublished data), we identified a pond [40.536314, -89.864681] at Banner Marsh State Fish and Wildlife Area (Fulton County, IL) known to contain Physa spp. and *P. trivolvis* snails infected with echinostomes (*Echinoparyphium* and possibly other echinostomes, such as *Echinostoma*, which has been found at nearby ponds) and which serves as a breeding site for multiple frog species. Snails within the pond are typically found either on the mud substrate or attached to submergent or emergent vegetation (J. Marino and J. Jones, personal observation). The pond is manmade and approximately rectangular (~18 × 53 m), with a maximal depth of ~2 m during the experiment. Permission to use the site was obtained through the Illinois Department of Natural Resources. The enclosures used were the same as those used in the laboratory experiment.

On Day 1, 16 sets of 5 *R. clamitans* tadpoles (Stage 25, Gosner 1960) were transported to the field site in 1 L plastic containers containing reverse osmosis-filtered water. We distributed 8 stakes (150 cm tall polyvinyl chloride (PVC) pipe, 1 cm diameter) within the pond, each inserted ~15 cm into the substrate and at similar depths (~1.2 m). Two sets of 4 stakes were located on the east and west sides of the pond, with stakes on each side ~10 m apart. Five tadpoles were transferred into each enclosure. Prior to the experiment, tadpoles were sorted so that those included in the experiment were of similar size and then randomly assigned to each enclosure. A polystyrene block (~2 × 2 × 10 cm) was also placed in each enclosure to provide buoyancy. Enclosures were tied with plastic zip ties and secured to the stake either just below the surface of the water or at 0.75 m depth (2 enclosures per stake × 5 tadpoles × 8 stakes = 80 tadpoles). On Day 11, we collected, sacrificed, and subsequently preserved all tadpoles from all cages in 70% ethanol. We measured snout-vent length (SVL) and *Gosner* (1960) stage for each
animal. To quantify infection load, we dissected the mesonephri, pronephri, and nephric ducts to quantify intrarenal metacercariae (i.e., echinostomes, as for the laboratory experiment) and also inspected the body and viscera for the presence of extrarenal metacercariae. In this case, we suspect that extrarenal metacercariae were most likely a plagiorchid, which result from cercariae that penetrate the tadpole’s skin (Rohr et al. 2010), due to their position in the host and the known presence of plagiorchids in the experimental pond (Steenrod et al., unpublished data). Data for any tadpoles that died during the experiment were not included in analyses. In addition, 1 tadpole survived the experiment but was degraded prior to dissection and is therefore included in analyses of survival but not infection or SVL.

Statistical analyses
We analyzed trematode infection load (number of metacercariae) and prevalence (proportion of tadpoles that were infected) in tadpoles from the laboratory and field experiments using generalized linear mixed effect models with a negative binomial distribution and a binomial distribution, respectively. For the laboratory experiment, we included a fixed effect of water column position (top cage, bottom cage, or free) and a random effect of stake. For the field experiment, we included a fixed effect of water column position (surface or near bottom) and a random effect of stake; as we observed both intra- and extrarenal metacercariae in the field, separate analyses were performed for intrarenal, extrarenal, and total metacercariae. We also examined how SVL (log-transformed to better meet assumptions of the statistical test) and survival differed depending on water column location in the field experiment using a general linear mixed effect model and a binomial generalized linear mixed effects model, respectively, with a random effect of stake. Likelihood ratio tests (LRT) were performed to determine the significance of model terms comparing a model including the water column predictor to a null model excluding that predictor.

Results
Laboratory experiment
One tank was excluded from analysis because no infection was observed, presumably because the snail produced few or no cercariae during the experiment duration. Tadpoles differed in final infection load depending on whether they were in a cage at the surface of the tank, caged at the bottom of the tank, or free. Free tadpoles experienced the highest infection load, with the lowest load observed in tadpoles enclosed in cages at the top of tanks (Figure 2: LRT: $\chi^2 = 18.93, df = 2, P < 0.001$). Tadpoles in cages on the bottom were on average infected with 83% more metacercariae than tadpoles in cages at the top. Prevalence (percent of tadpoles that experienced infection) was significantly higher in free (100% prevalence) and bottom-enclosed (87% prevalence) tadpoles compared with surface-enclosed tadpoles (47% prevalence; $\chi^2 = 12.30, df = 3, P = 0.0015$). A comparison excluding the free tadpoles from the analysis indicated that the difference in infection load between tadpoles in cages at the bottom or top of tanks was marginally non-significant (LRT: $\chi^2 = 3.79, df = 1, P = 0.052$), whereas the difference in prevalence was significant ($\chi^2 = 6.12, df = 1, P = 0.013$). There was no mortality across all tanks and cages.

Field experiment
We were unable to recover cages from one stake, limiting analyses to 7 replicates. Furthermore, upon collection, 2 enclosures were found to have small holes, via which tadpoles or potential predators (e.g., dragonfly larvae) possibly could have moved in or out of the enclosures; we therefore excluded those enclosures from our analyses.

Whether combining both types of metacercariae or analyzing them separately, tadpoles in cages at the surface experienced lower infection loads than those in cages deeper in the water column (Figure 3A and B; LRT: total metacercariae 730% higher in bottom cages; $\chi^2 = 40.27, df = 1, P < 0.001$; intrarenal metacercariae 331% higher in bottom cages; $\chi^2 = 18.56, df = 1, P = 0.004$; extrarenal metacercariae 162.4% higher in bottom cages; $\chi^2 = 46.34, df = 1, P < 0.001$). Prevalence of infection with both intrarenal and extrarenal metacercariae was 100%, whereas prevalence of infection in near-surface cages was significantly lower (Figure 3C and D; intrarenal: $\chi^2 = 8.81, df = 1, P = 0.0030$; extrarenal: $\chi^2 = 20.68, df = 1, P < 0.001$)—64% and 41% for intra- and extrarenal metacercariae, respectively.

We found 62.9% (22/35 tadpoles) and 76.0% (19/25 tadpoles) survival in top and bottom enclosures, respectively (Figure 4A);
there was no significant difference in survival across depths (LRT: $\chi^2 = 0.89, df = 1, P = 0.34$). SVL of tadpoles in cages at the surface were larger than tadpoles in deeper cages (Figure 4B, $\chi^2 = 6.04, df = 1, P = 0.014$). However, post-hoc analyses of infection incorporating SVL as a covariate did not indicate an association between SVL and infection load ($P > 0.1$) and suggest that variation in SVL did not influence the results of either the lab or field experiment. All tadpoles were at Gosner Stage 25 at the end of the experiment except 1 individual at Stage 27.

**Discussion**

Our results indicated that trematode infection of larval anurans depends on vertical position in the water column. The laboratory experiment suggested a pattern in infection occurs with depth, with 83% higher infection loads and 85% higher prevalence near the bottom of the water column, although the difference in load was marginally non-significant, potentially due to the limited sample size ($n = 5$) and shorter duration compared with the field study. In the field, a similar but more striking pattern occurred, as tadpoles experienced significantly greater infection loads and prevalence with both echinostome (intrarenal) and extrarenal metacercariae when deeper in the water column (0.75 m) compared with just below the surface.

One potential explanation for the observed depth-dependent infection risk is cercaria phototaxis and vertical movement. For example, a study in which cercariae of multiple trematode species were exposed to light of varying intensity and direction found that, regardless of light intensity, cercariae of the echinostome, *Echinostoma revolutum*, tend to swim away from a light source (Loy et al. 2001). Other studies of echinostomes have similarly shown negative phototaxis, particularly as cercaria age and infectivity increases (McCarthy 1999; Platt and Dowd 2012). In ponds, therefore, light entering at the water’s surface could serve to repel echinostome cercariae downward, aggregating them deeper in the water column and presumably increasing infection risk for hosts at greater depths. Similarly, another study found that for two plagiorchid species, aggregation toward the bottom of the water column was typical unless the water was made to be turbulent (McCarthy et al. 2002). Our results build on these previous studies by demonstrating that such parasite behaviors likely have consequences for transmission, even in field conditions.

A second proposed explanation for the observed difference in infection risk between depths is uneven vertical distribution of the first intermediate hosts, snails. Increased densities of mollusk first intermediate hosts at greater depths may translate into increased risk of trematode infection for second intermediate hosts, such as tadpoles, at those depths. Many species of aquatic snail, for instance, tend to occur relatively deep in the water column (Boag et al. 1984; Marklund et al. 2001), including *Planorbbella* and *Physa* (Boag 1981), which could in turn lead to higher densities of cercariae at greater depths and may thus have contributed to the observed patterns in infection.

The observed change in infection risk with depth likely has consequences for infection rates experienced by hosts in nature. Tadpoles frequently occupy the bottom of the water column, as they are scrapers that consume periphyton on the substrate and also may move deeper to avoid predators (Relvey and Werner 1999). Our results suggest that this use of deeper microhabitats may come at a cost of greater risk from parasite infection, which could have important implications for host–parasite dynamics. Furthermore, such effects are unlikely to be unique to this system. For other parasite taxa besides trematodes (e.g., nematodes, acanthocephalans), factors (e.g., light, temperature) that vary with depth or height may similarly mediate the movement of free-living infective stages (Sciacca et al. 2002; Tanaka et al. 2013) or intermediate hosts.

![Figure 3. Number of (A) intrarenal (echinostome) or (B) extrarenal cysts (metacercariae) and prevalence of infection with (C) intrarenal and (D) extrarenal cysts in *Rana clamitans* tadpoles in either near-surface enclosures (7 enclosures, 22 tadpoles) or near-bottom enclosures (5 enclosures, 18 tadpoles) on Day 11 of the field experiment. Lower case letters above boxes indicate significant differences ($P < 0.05$).](image-url)
Another implication of our results is that variation in infection risk could influence host behavior. Although we did not measure behavior here, we previously observed an increase in the proportion of tadpoles near the water surface in response to the presence of cercariae (Marino 2016a). Our findings here indicating vertical variation in infection risk support the hypothesis that this vertical host movement reflects an adaptive avoidance response by tadpoles to reduce exposure and infection. Our results are thus in line with other studies which have detected spatial avoidance behavior in larval amphibians in response to trematodes (e.g., Poulin and Fitzgerald 1989; Jokela et al. 1999). Overall, our study thus adds new insight into the complex system of factors that govern natural enemy interactions in space.

An unexpected result was that the free tadpoles in the laboratory experiment experienced the highest infection loads compared with tadpoles in enclosures, despite our expectation that the free tadpoles would be better able to avoid parasites. There may be a number of reasons why we observed this pattern; cages may have deterred cercariae from infecting caged tadpoles or free tadpoles may have encountered parasites at a greater rate as a result of greater freedom to swim around the tanks (e.g., Wilson et al. 1993). In addition, the food for both snails and tadpoles typically sank to the bottom of the tanks, so that free tadpoles may have spent more time near the bottom than if food was evenly distributed across depths.

One additional consideration is that body size can influence infection. Increased body size is known to correlate with higher infection load in both field (Marino et al. 2017) and experimental (Marino et al. 2016) settings; therefore, the larger size of tadpoles in the cages near the water surface is unlikely to explain the observed pattern in infection in our field experiment. It is possible, however, that the higher infection loads observed when tadpoles were deeper resulted in higher energetic costs, which could have contributed to lower growth rates during the experiment (Orlofske et al. 2017).

In summary, we have presented novel evidence of a link between water column position and parasite infection risk in the larval amphibian-trematode system, which was generally consistent across field and laboratory settings. Due to the close association between spatial distribution and behavior, this finding has implications for anti-parasite behavior (Marino 2016a) and is likely relevant in other vertically structured host–parasite systems (e.g., Poulin and Fitzgerald 1989; Jokela et al. 1999). Overall, our study thus adds new insight into the complex system of factors that govern natural enemy interactions in space.

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Authors’ Contributions
J.A.M, J.R.J., and C.L.S. conceived the hypotheses and experimental design; J.R.J. and C.L.S. collected the data; J.R.J. and J.A.M. analyzed data; J.R.J. led the writing of the manuscript; all authors contributed critically to the drafts and gave final approval for publication.

Data Archiving
Data available from the Dryad Digital Repository: (https://doi.org/10.5061/dryad.631j930).

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