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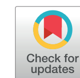
Parasite susceptibility in an amphibian host is modified by salinization and predators☆



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ABSTRACT

Secondary salinization represents a global threat to freshwater ecosystems. Salts, such as NaCl, can be toxic to freshwater organisms and may also modify the outcome of species interactions (e.g. host-parasite interactions). In nature, hosts and their parasites are embedded in complex communities where they face anthropogenic and biotic (i.e. predators) stressors that influence host-parasite interactions. As human populations grow, considering how anthropogenic and natural stressors interact to shape host-parasite interactions will become increasingly important. We conducted two experiments investigating: (1) the effects of NaCl on tadpole susceptibility to trematodes and (2) whether density- and trait-mediated effects of a parasite-predator (i.e. damselfly) and a host-predator (i.e. dragonfly), respectively, modify the effects of NaCl on susceptibility to trematode infection. In the first experiment, we exposed tadpoles to three concentrations of NaCl and measured parasite infection in tadpoles. In the second experiment, we conducted a 2 (tadpoles exposed to 0 g L^{-1} NaCl vs. 1 g L^{-1} NaCl) x 4 (no predator, free-ranging parasite-predator (damselfly), non-lethal host-predator (dragonfly kairomone), and free-ranging parasite-predator + dragonfly kairomone) factorial experiment. In the absence of predators, exposure to NaCl increased parasite infection. Of the predator treatments, NaCl only caused an increase in parasite infection in the presence of the parasite-predator. However, direct consumption of trematodes caused a reduction in overall infection in the parasite-predator treatment. In the dragonfly kairomone treatment, a reduction in tadpole movement (i.e. trematode avoidance behavior) led to an increase in overall infection. In the parasite-predator + dragonfly kairomone treatment, antagonistic effects of the parasite-predator (reduction in trematode abundance) and dragonfly kairomone (reduction in parasite avoidance behavior) resulted in intermediate parasite infection. Collectively, these findings demonstrate that NaCl can increase amphibian susceptibility to parasites, and underscores the importance of considering predator-mediated interactions in understanding how contaminants influence host-parasite interactions.

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1. Introduction

Chemical contaminants represent one of the greatest current threats to aquatic ecosystems (Cañedo-Argüelles et al., 2013; Chagnon et al., 2015; Lesbarrères et al., 2014; Sasaki et al., 2015). Of particular concern is the unprecedented rate of salt accumulation in coastal and inland freshwater ecosystems (i.e. secondary salinization; Herbert et al., 2015). Salts can enter freshwater habitats through agricultural practices (i.e. irrigation water runoff), coastal flooding, and road salt application (Hill and Sadowski, 2016). Secondary salinization of freshwater systems is predicted to continue increasing over time because unlike some contaminants that degrade rapidly, the high environmental stability of salts result in their accumulation within water and soil (Herbert et al., 2015). Therefore, understanding the ecological consequences of salinization is critical to protecting our freshwater ecosystems.

Salinization of freshwater systems can result in both direct and indirect effects on aquatic organisms. Single-species toxicity assays have demonstrated that salt concentrations commonly found in nature are directly toxic to a number of freshwater species (Sanzo and Hecnar, 2006; Karraker et al. 2008; Mahrosh et al., 2014; Searle et al., 2016). For example, Collins and Russell (2009) found that Cl^{-} concentrations that are commonly found in roadside

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wetlands ($0.3–6.1\ g\ L^{-1}$) caused mortality across five common North American amphibian species. In addition to the direct toxic effects of salt on individual organisms, increased salinization can negatively affect aquatic organisms via indirect pathways. Using low, environmentally-relevant concentrations of NaCl, Hintz et al. (2016) found that NaCl caused a reduction in the density of zooplankton, resulting in a direct positive effect of salinity on phytoplankton abundance. This increase in phytoplankton abundance ultimately resulted in a trophic cascade that lowered the biomass of filamentous algae. As secondary salinization continues to threaten freshwater systems, studies evaluating both the direct and indirect effects of salt on species interactions will be critical for understanding the mechanisms that shape species abundance and diversity.

The effect of contaminants on the interaction between hosts and their parasites has received significant recent attention (Gustafson, 2016; Sures et al., 2017). Environmental contaminants can mediate host-parasite interactions via several mechanisms. For example, contaminants can influence host-parasite interactions directly by reducing the abundance of either parasite or host (Rohr et al. 2008a,b) or by altering their respective traits, such as infectivity of the parasite, immune response of the host, behavior of the parasite, or behavior of the host (Christin et al., 2003; Rohr et al. 2008a,b; Denoël et al., 2013). Despite the influence that contaminants may have on host-parasite interactions, relatively few studies have investigated the consequences of salinization on disease outcomes (Hall et al., 2013; Milotic et al., 2017; Studer and Poulin, 2012). Additionally, salinization can often occur at relatively low concentrations ($\leq 1\ g\ L^{-1}$ NaCl; Milotic et al., 2017). While these low concentrations of salt may not cause direct mortality (Gonçalves et al., 2007; Collins and Russell, 2009), it is imperative to consider the potential indirect effects that these low concentrations may have on host-parasite interactions.

Finally, in nature, hosts and their parasites are embedded in complex ecological communities where they interact with other species that can also contribute to the effects of contaminants on disease outcomes. For instance, the presence of predators can directly alter infection outcomes by reducing abundance of the parasite or host (a density-mediated effect; Ostfeld and Holt, 2004; Orlofske et al., 2014), as well as influence infection outcomes by altering the behavior of hosts, such as those that are associated with parasite avoidance. For example, in the presence of trematodes, tadpoles commonly display heightened activity levels to avoid trematode infection (Daly and Johnson, 2011). However, in the presence of both predators and trematodes, tadpoles have been shown to reduce their activity levels to avoid predation, leading to an increase in trematode infection (a trait-mediated effect; Thiemann and Wassersug, 2000; Orlofske et al., 2014). Despite the potential role that other species (i.e. predators) in the community may play in shaping disease outcome, most studies investigating the effects of contaminants on host-parasite outcomes are conducted in isolation of other species interactions (Koprivnikar et al., 2007; Rohr et al. 2008a,b; Studer and Poulin, 2012; Milotic et al., 2017). Hosts and their parasites encounter both abiotic (i.e. contaminants) and biotic factors (i.e. predators) within their natural environment. Therefore, to better understand how contaminants affect host-parasite outcomes, there is a need to consider the added complexity of interactions between multiple factors.

Towards this goal, an amphibian-trematode model is an excellent system for testing the interaction of salinization and predators on host-parasite interactions. Many have hypothesized that contaminants play a significant role in shaping disease outcomes of amphibians (Collinge and Ray, 2006; Rohr et al. 2008a,b). Indeed, previous work demonstrates that both amphibians and cercariae, a free-swimming stage in the trematode life cycle, are susceptible to environmental contaminants (i.e. pesticides) at ecologically-relevant concentrations (Rohr et al. 2008a,b; Hua et al., 2016). Further, this system is also ideal because past work has shown that contaminants and predators can both independently mediate infection outcomes in host-parasite interactions (Rohr et al. 2008a,b, 2015, Orlofske et al., 2012, 2014, Groner and Relyea, 2015). Therefore, using the amphibian-trematode model, this study aimed to: 1) Determine the effects of increased salinity on tadpole susceptibility to trematode infection 2) Investigate whether density-mediated effects of a parasite-predator and trait-mediated effect of a non-lethal host-predator can modify the effects of increased salinity on infection outcomes.

2. Materials and methods

2.1. Study system

To address these goals, we chose to work with environmentally-relevant concentrations of NaCl. NaCl is a ubiquitous wetland contaminant which can enter freshwater systems in a variety of ways. In northern latitudes, NaCl is found within freshwater largely as a result of road deicer runoff (Evans and Frick, 2001; Kelly et al., 2010). Chloride concentrations associated with road de-icing have been shown to range from $0.150\ g\ L^{-1}$ in rural lakes to as high as $5\ g\ L^{-1}$ in more urban lakes and wetlands (Evans and Frick, 2001). Further, NaCl can also enter wetlands through the clearing of natural vegetation for agriculture by reducing buffer zones between roadways and wetlands (Barica, 1972). Other agricultural practices such as crop irrigation can also increase wetland salinization, as water used to irrigate crops often contains naturally occurring salts (Ghassemi et al., 1995). Chloride concentrations in ground water near agricultural areas can be as high as $46\ g\ L^{-1}$ due to these irrigation practices, and can then leach or runoff into nearby bodies of water (Herbert et al., 2015).

For the parasite, we chose to work with echinostomes, a family of digenetic trematodes. Trematodes have complex life cycles, with snails and bivalves serving as the first intermediate host, amphibian larvae as the second intermediate host, and birds or mammals serving as definitive hosts (Huffman and Fried, 2012). As the first intermediate host, snails are penetrated by miracidia in the head-foot region, which forms a sporocyst (Kanev et al., 2000). Sporocysts then produce rediae, which form free-living cercariae. These cercariae leave the snail, infect amphibian tadpoles, and then encyst in the kidneys of the tadpole forming metacercariae. Amphibian hosts are consumed by bird or mammalian predators, where the parasite sexually reproduces within the gut of the bird or mammalian host. The parasites then return to the aquatic environment via eggs embedded in the feces of the bird or mammalian host. Trematode infections are the most common parasite infection reported across several amphibian species, with mortality and pathology of amphibian hosts being dose-dependent (increasing with total number of metacercariae within the host; Holland et al., 2007). We utilized the cercarial stage of the trematode, as it is one of the stages that is most likely to be directly exposed to NaCl within wetlands. This free-living cercariae stage also serves a vital role as a prey species within several aquatic food webs (Johnson et al., 2010; Lafferty et al., 2006).

For the host, we chose to work with wood frog tadpoles (*Lithobates sylvaticus*). Wood frogs are one of the most widely distributed amphibian species (Conant and Collins, 1998) and often occur in bodies of water that are located near roadways where they may be exposed to NaCl (Sanzo and Hecnar, 2006; Karraker et al. 2008). Additionally, they have been shown to be susceptible to both NaCl ($LC50_{96} = 5.11\ g\ L^{-1}$ NaCl; Sanzo and Hecnar, 2006) and to parasite infection (Rohr et al. 2008a,b; Pochini and Hoverman, 2017).

Further, wood frog tadpoles have been shown to respond plastically to predator cues by altering their activity in the presence of caged predators (Schoeppner and Relyea, 2009).

Finally, to assess whether predators alter the consequences of NaCl on infection outcomes, we used two common odonate nymphs (damselfly- *Enallagma* sp. and dragonfly- *Anax junius*). These odonates are ideal organisms for isolating density- and trait-mediated effects of predators on amphibian–trematodes interactions. For example, *Enallagma* sp. are predators of cercariae but are not predators of amphibian larvae. *A. junius* are predators of tadpoles and while they are capable of consuming cercariae (Rohr et al., 2015), we used kairomones to prevent direct predation of tadpoles and cercariae by *A. junius*. This study system serves as a model to test the effects of an environmental contaminant on host-parasite outcomes, while also considering the added realistic complexity of density-and-trait-mediated effects of parasite and amphibian predators.

2.2. Animal collection and husbandry

On 12 March 2016, we collected 10 partial wood frog egg masses (Gosner stage 4 (Gosner, 1960); from four populations in western Pennsylvania, USA, for a total of 40 individual egg masses. We placed egg masses from each population into separate plastic wading pools filled with 255 L of well-water (five egg masses/pool). Once animals reached Gosner stage 25 (Gosner, 1960), they were fed rabbit chow *ad libitum* until the start of each experiment.

To obtain cercariae for the experiments, on 29 March and 20 June 2016, we collected 120 and 80 *Helisoma trivolvis* from the Purdue Wildlife Area (West Lafayette, IN) and Harpur Pond (Binghamton, NY), respectively. We screened all snails for trematode infection by placing individuals into 50 mL Falcon tubes filled with 35 mL aged well-water under a heat lamp for 1 h to induce the shedding of cercariae (Cohen et al., 1980). We identified trematode cercariae to the family level (Family Echinostomatidae) through morphological observations (Gibson et al., 2005). Once we identified infected snails, to limit further cercarial shedding, we housed snails in 14 L containers filled with 10 L of aged well-water at 4 °C until the day before each experiment.

We collected 60 damselfly nymphs on 20 June 2016 from Harpur Pond (Binghamton, NY). Damselfly nymphs were kept at 21 °C in 14 L containers filled with 10 L of aged well-water and were fed an assemblage of zooplankton, primarily *Scapholeberis mucronat*a and *Daphnia pulex* from stock lab cultures, *ad libitum*. We conducted water changes every three days.

We collected 10 dragonfly nymphs on 22 June 2016, from Nuthatch Hollow (Binghamton, NY). Dragonfly nymphs were housed separately in 1 L containers, filled with 700 mL of aged well-water at 21 °C. We fed the dragonfly nymphs a single wood frog tadpole every other day and conducted water changes every three days.

2.3. NaCl solution concentrations and testing

To create working solutions, we used laboratory grade NaCl (>99% NaCl, Baker Scientific) dissolved in aged well-water. We measured NaCl concentrations using a multi-parameter Sonde (YSI, Ohio, USA). Nominal concentrations used in Experiment 1 were 0, 1, 2, and 3 $g\,L^{-1}$, with actual concentrations being 0, 1.05, 2.14, and 3.21 $g\,L^{-1}$, respectively. Nominal concentrations used in Experiment 2 were 0 and 1 $g\,L^{-1}$, with actual concentrations being 0 and 1.04 $g\,L^{-1}$, respectively. Thus, actual concentrations were all within 7% of their respective nominal concentrations.

2.4. Experiment 1: exposure of tadpoles to NaCl and cercariae

In this experiment, we examined the effects of NaCl on wood frog susceptibility to parasite infection. On 24 June 2016, we haphazardly selected 60 tadpoles across all four populations to ensure genetic variation within our experiment. From this subsample, we exposed tadpoles to NaCl treatments by distributing tadpoles into each of our four NaCl treatments (0, 1, 2, and 3 $g\,L^{-1}$ NaCl; Average tadpole mass ± Standard error; 157.51 ± 5.35 mg). After 24 h, we transferred all tadpoles from each exposure treatment into our experimental units, which were 100 mL plastic cups filled with 60 mL of aged NaCl-free well-water.

On 24 June, we moved five infected snails from the Purdue Wildlife Area population that were being held at 4 °C into new 14 L tubs filled with 10 L of aged well-water to acclimate for 24 h at 21 °C. On 25 June, to obtain cercariae for the experiment, we placed infected snails in separate 50 mL Falcon tubes filled with 35 mL of aged well-water and exposed them to a heat lamp for 1 h to induce cercarial shedding (Cohen et al., 1980). After shedding cercariae from each snail, we mixed cercariae from each Falcon tube into a single petri dish. We then pipetted 50 cercariae into 60 Falcon tubes filled with 5 mL of aged well-water ($N = 3000$ total cercariae). We then poured the entire content of a single Falcon tube into each of our 60 experimental units, rinsing tubes three times using water from the respective unit to ensure that all cercariae were added. Previous work has shown that cercariae infectivity can change depending on the age of the cercariae (Pechenik and Fried, 1995). However, these studies, as well as our own pilot work found that infectivity is consistent in cercariae that are 0–5 h old. Therefore, to control for cercariae age and infectivity, we completed our infection procedures within 2 h.

We terminated the experiment 24 h after adding tadpoles to units as this is an adequate amount of time for cercariae to encyst within the tadpole hosts (Rohr et al. 2008a,b) and before metacercariae clearance begins (Pochini and Hoverman, 2017; Rohr et al. 2008a,b). We euthanized all tadpoles with an overdose of MS-222 and then preserved all individuals in 10% formalin. We measured Gosner stage (Gosner, 1960), mass, and snout-vent-length (SVL) for each individual. To investigate parasite infection, we dissected each tadpole beneath a dissecting microscope and removed their kidneys. We then placed the kidneys between microscope slides and then scanned the slides under a dissecting microscope, recording the number of metacercariae present (Rohr et al. 2008a,b). We additionally scanned the body cavity to ensure that all metacercarial cysts were counted.

2.5. Experiment 2: effects of NaCl and predators on parasite infection

To investigate how trait-and-density-mediated effects of predators modify the effects of NaCl on parasite infection we conducted a 2 (tadpole exposed to 0 $g\,L^{-1}$ NaCl vs. 1 $g\,L^{-1}$ NaCl) x 4 (no predator, free-ranging parasite-predator (damselfly), non-lethal host-predator (dragonfly kairomone), or combination of both free-ranging parasite-predator and non-lethal host-predator factorial design, replicating each treatment 10 times for a total of 80 experimental units. In Experiment 2, because we were interested in assessing trait-mediated effects, we increased the volume of water in the experimental units relative to Experiment 1 to allow tadpoles more room to display trait-mediated responses (i.e. anti-predator/parasite behavior). Therefore, experimental units were 2 L plastic containers filled with 1 L of aged well-water with an average density of 54 ± 2.9 SE cercariae. Similar to Experiment 1, to prevent direct effects of NaCl on cercariae, we chose to conduct our experiment in NaCl-free aged well-water. Thus, tadpoles were

exposed to NaCl, while cercariae were not.

Tadpole exposure to NaCl— On 25 June 2016, we haphazardly selected 20 tadpoles from each of our four populations and placed them into 10 L of aged well-water. We then size-selected and distributed the tadpoles into plastic tubs filled with 10 L of aged well-water (no NaCl exposure) or 10 L of 1 g L^{-1} NaCl (NaCl exposure; Average tadpole mass ± SE = 141.52 ± 13.35 mg; Density = 2 tadpoles L^{-1}). We chose to expose tadpoles to 1 g L^{-1} as it is an environmentally-relevant NaCl concentration (Milotic et al., 2017). After 24 h, keeping the treatments separate, we placed tadpoles into 4 new tubs with 10 L of NaCl-free aged well-water until the start of the experiment (26 June).

Kairomone addition— On 26 June, we size-selected five dragonfly nymphs for collection of kairomones. We placed each dragonfly nymph into separate 2 L plastic containers filled with 1 L of aged well-water. We then fed each dragonfly nymph five wood frog tadpoles over a 1 h period. After each dragonfly consumed five wood frog tadpoles, we removed the dragonfly nymph and combined the contents of each 1 L plastic container for a total of 5 L of dragonfly kairomone (5 consumed tadpoles L^{-1}; average tadpole mass ± SE = 145 ± 13 mg).

We then added 25 mL of this cue to each of the dragonfly kairomone and free-ranging parasite-predator + dragonfly kairomone experimental units. We mock-dosed units not in the dragonfly kairomone treatment by adding 25 mL of aged well-water to each of these units.

Damselfly addition— We size-selected 20 damselflies (Mass ± SE; 0.0138 ± 0.0033 mg) and placed a single individual into each of the free-ranging parasite-predator, and free-ranging parasite-predator + dragonfly kairomone experimental units. Damselflies were placed into experimental units 30 min prior to the start of the experiment to allow individuals to acclimate.

Addition of cercariae— To obtain cercariae for the experiment, we placed eight infected snails from the Harpur Pond population in separate 50 mL Falcon tubes filled with 35 mL of aged well-water, and exposed them to a heat lamp for 1 h to induce cercarial shedding (Cohen et al., 1980). We then combined the contents of all eight tubes into a single glass beaker. We calculated the number of cercariae/mL by pipetting 2 mL from the glass beaker containing cercariae and counting the number of cercariae in each sample. We repeated this methodology five times and determined that there was on average 10.8 ± 0.58 SE cercariae mL^{-1}. To achieve an approximate density of 50 cercariae per experimental unit, we pipetted 5 mL of this cercariae mixture into 80, 50 mL Falcon tubes filled with 5 mL of water (Tucker et al., 2001; Wuerthner et al., 2017). We then poured the entire contents of a single Falcon tube into each of our 80 experimental units, rinsing tubes three times using water from the respective unit to ensure that all cercariae were added. Due to an increase in experimental units in Experiment 2, we used this method of counting cercariae as opposed to the method used in Experiment 1, to ensure that cercariae were added to experimental units within 2 h of emergence from their snail hosts. This allowed us to control for cercarial age between our treatments and between the two experiments.

Addition of tadpoles— Finally, we added a single tadpole that was previously exposed to one of the two NaCl exposure treatments (0 g L^{-1} vs. 1 g L^{-1}) to each experimental unit. The addition of the tadpole to the experimental unit marked the start of the experiment. To prevent the damselfly from consuming cercariae prior to the start of the experiment, we added tadpoles to their respective experimental units immediately after the addition of cercariae.

2.6. Response variables

To understand how trait and density-mediated effects of predators modify the effects of NaCl on parasite infection we assessed and compared tadpole activity and parasite infection (# of metacercariae in tadpole kidneys) in each of the eight treatments. Tadpole activity is an important measure as parasite infection has been shown to increase with decreased tadpole activity (Milotic et al., 2017; Orlofske et al., 2014).

Tadpole activity assays— To examine the effects of a free-ranging parasite-predator, dragonfly kairomone, the combination of both free-ranging parasite-predator and dragonfly kairomone, and prior exposure to NaCl on tadpole activity, we conducted two activity assays to assess tadpole movement. We conducted these activity assays at the same time as the main infectivity experiment. In the first assay, we defined activity as the number of times the tadpole's snout crossed standardized gridlines that we placed beneath each unit (Fig. 1). We conducted the first assay 30 s after the tadpole was added to a unit by counting the number of times the tadpoles crossed standardized gridlines for 1 min. We conducted the second assay 3 h after the start of the experiment by quantifying tadpole movement using scan sampling (Relyea and Mills, 2001). For the scan sampling assay, we observed each experimental unit 20 times over the course of 30 min and recorded whether tadpoles were moving.

Parasite infection— We terminated the experiment 24 h after adding tadpoles to units and euthanized tadpoles using an MS-222 overdose. We then preserved all tadpoles in 10% formalin. We measured Gosner stage (Gosner, 1960), mass, SVL and parasite infection for each individual. To quantify parasite infection, we used the same methodology described for Experiment 1.

2.7. Statistical analysis

2.7.1. Experiment 1: effects of NaCl on parasite infection

Parasite infection - We used a generalized linear model (GLM;

Fig. 1. Image showing experimental unit used for tadpole activity assay containing tadpole host, free-ranging parasite-predator (damselfly), and non-lethal host-predator (kairomone). We quantified activity by counting the number of times the tadpole's snout passed a gridline for a 1-min period.

McCullagh and Nelder, 1989) with a Poisson distribution and log-link function to investigate the main effects of tadpole exposure to NaCl on parasite infection. We chose this statistical model because our response variables were count data (O'Hara and Kotze, 2010; Warton and Hui, 2011), and because of their flexibility with data that have varying error distributions (Nelder and Baker, 1972). We included tadpole Gosner stage (Gosner, 1960) as a covariate in our analysis, as Gosner stage has been shown to be correlated with susceptibility to parasite infection (Johnson et al., 2011; Rohr et al., 2010). We could not quantify the number of metacercariae in one individual from the 1 g L^{-1} NaCl treatment due to sample degradation, so we dropped this individual from our analysis.

2.7.2. Experiment 2: effects of NaCl and predators on parasite infection

Tadpole activity assays – For the activity assay we used a GLM with a negative binomial error distribution and power function as our data were over dispersed (White and Bennetts, 1996) to investigate the main effects of NaCl exposure, predator treatment, and their interaction, on tadpole activity. For the scan assay we used a GLM with a Poisson distribution and log-link function. For significant main effects and interactions, we conducted Bonferroni-adjusted planned contrast for both activity assays (Quinn and Keough, 2002).

Parasite infection – We used a GLM with a Poisson distribution and log-link function to investigate the main effects of tadpole exposure to NaCl, predator treatments, and their interaction on parasite infection. Similar to Experiment 1, we included tadpole Gosner stage (Gosner, 1960) as a covariate in our analysis, as Gosner stage has been shown to be correlated with susceptibility to parasite infection (Johnson et al., 2011; Rohr et al., 2010). For significant main effects or interactions, we conducted a Bonferroni-adjusted planned contrast (Quinn and Keough, 2002). All data were analyzed using IBM SPSS software (Version 22, IBM, INC).

3. Results

3.1. Experiment 1: effects of NaCl on parasite infection

We found a significant main effect of NaCl exposure on tadpole susceptibility to parasite infection ($\chi^2 = 73.0$; $p < 0.001$; Fig. 2; Appendix Table 1A). Relative to tadpoles not exposed to NaCl, exposure to 1, 2, and 3 g L^{-1} of NaCl increased parasite infection by 68%, 63%, and 96%, respectively ($p < 0.001$ for all comparisons). Tadpoles exposed to 3 g L^{-1} NaCl treatment had 17% and 20% more parasites compared to tadpoles in the 1 g L^{-1} ($p = 0.033$) and 2 g L^{-1} NaCl ($p = 0.010$) treatments, respectively. Finally, there was no difference in parasite infection between tadpoles exposed to 1 g L-1 vs. 2 g L^{-1} NaCl ($p = 0.680$).

3.2. Experiment 2: effects of NaCl and predators on parasite infection

Tadpole activity assays- We found a significant overall effect of predator and NaCl treatments on tadpole activity ($\chi^2 = 20.5$; $p = 0.005$; Fig. 3; Appendix Table 2A). We found a significant main effect of predator treatment on tadpole activity ($\chi^2 = 14.7$; df = 3; $p < 0.001$) but no significant main effect of exposure to NaCl ($\chi^2 = 2.2$; df = 1; $p = 0.650$) and no significant interaction between predator and NaCl treatments ($\chi^2 = 2.2$; df = 3; $p = 0.523$). Relative to tadpoles in the no predator treatment, tadpole activity was 53% lower in the free-ranging parasite-predator treatment (damselfly; $p = 0.035$), 75% lower in the non-lethal host-predator treatment (dragonfly kairomone; $p = 0.004$), and 57% lower in the free-ranging parasite-predator + dragonfly kairomone combination

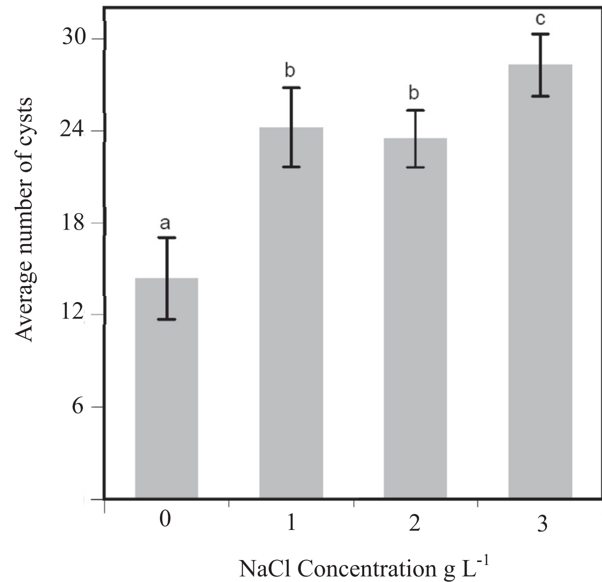

Fig. 2. Number of cercariae encysted in tadpoles (average ± SE) that were previously exposed to 0, 1, 2, or 3 g L^{-1} NaCl. Treatments sharing lower case letters are not significantly different from each other ($p > 0.05$).

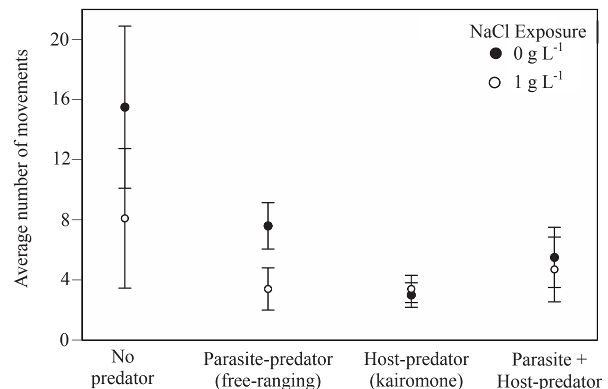

Fig. 3. Number of times wood frog tadpoles moved across a gridline (average ± SE) across salinity (exposed to either 0 or 1 g L^{-1} NaCl) and predator treatments (no predator, free-ranging parasite-predator (damselfly), non-lethal host-predator (dragonfly kairomone), and combination of both free-ranging parasite-predator and dragonfly kairomone treatments.

treatment ($p = 0.035$). Tadpole activity did not differ between any of the predator treatments ($p > 0.05$). We report all pairwise comparisons for tadpole activity data in Appendix Table 1B.

We also found a significant overall effect of predator and NaCl treatment on tadpole activity from our scan sampling assay ($\chi^2 = 14.6$; $p = 0.042$). We found a significant main effect of predator presence ($\chi^2 = 11.2$; df = 3; $p = 0.011$), but not NaCl exposure ($\chi^2 = 0.2$; df = 1; $p = 0.643$), and no significant interaction between predator and NaCl treatments ($\chi^2 = 2.8$; df = 3; $p = 0.420$) on tadpole activity. Because results were similar to the initial activity assay, we report the pairwise comparisons in Appendix Table 1C.

Parasite infection- We found a significant overall effect of predator and NaCl treatments on tadpole susceptibility to parasite infection ($\chi^2 = 134.9$; $p < 0.001$; Fig. 4.; Appendix Table 3A). We found a significant main effect of predator treatment ($\chi^2 = 101.7$; df = 3; $p < 0.001$), a main effect of NaCl treatment ($\chi^2 = 16.6$; df = 1; $p < 0.001$), and an interaction between predator and NaCl treatments ($\chi^2 = 33.4$; df = 3; $p < 0.001$) on parasite infection. To

facilitate the description of the predator by NaCl interaction, for each predator treatment, we report the pairwise analyses comparing number of parasites encysted in tadpoles not exposed to NaCl versus those exposed to NaCl.

In the predator-free treatment, tadpoles exposed to NaCl had 170% more parasites encysted than individuals not exposed to NaCl ($p < 0.001$). Similarly, in the free-ranging parasite-predator treatment, tadpoles exposed to NaCl had 200% more parasites encysted compared to tadpoles not exposed to NaCl ($p = 0.003$). In contrast, for the dragonfly kairomone and parasite-predator + dragonfly kairomone combination treatments, exposure to NaCl did not significantly affect tadpole susceptibility to parasites ($p = 1.000$; $p = 0.700$, respectively). For all other pairwise comparisons refer to Table 2B in the Appendix.

4. Discussion

In this study, we examined the effects of environmentally-relevant concentrations of NaCl on tadpole susceptibility to parasite infection. Additionally, we investigated the role that predators play in altering host-parasite outcomes via trait-and-density-mediated effects after tadpoles were exposed to NaCl. We found that NaCl increased parasite infection in tadpole hosts. However, the effects of NaCl on host-parasite outcomes were modified by both parasite and host-predators. Ultimately, this illustrates the need for studies to incorporate the interaction between abiotic (i.e. contaminants) and biotic stressors (i.e. predators) when examining the impacts of contaminants on host-parasite dynamics.

In Experiment 1, we demonstrated that exposure to environmentally-relevant concentrations of NaCl (1, 2, and $3\,g\,L^{-1}$) increased parasite infection relative to control individuals. Other studies have shown similar effects on amphibian-trematode interactions using different environmental contaminants. For instance, Rohr et al. (2008) demonstrated that environmentally-relevant concentrations of four common pesticides increased parasite infection within tadpole hosts. Past work suggests that a contaminant-induced reduction in tadpole movement is one potential mechanism driving increased susceptibility of exposed tadpoles to cercariae (Taylor et al., 2004). Specific to NaCl, past work demonstrates that wood frog tadpoles exposed to 0.00039, 0.07750, and $1.030\,g\,L^{-1}$ NaCl had reduced movement to stimuli and had difficulty swimming after being exposed to NaCl for 5 days. In our study, tadpoles exposed to NaCl had increased parasite

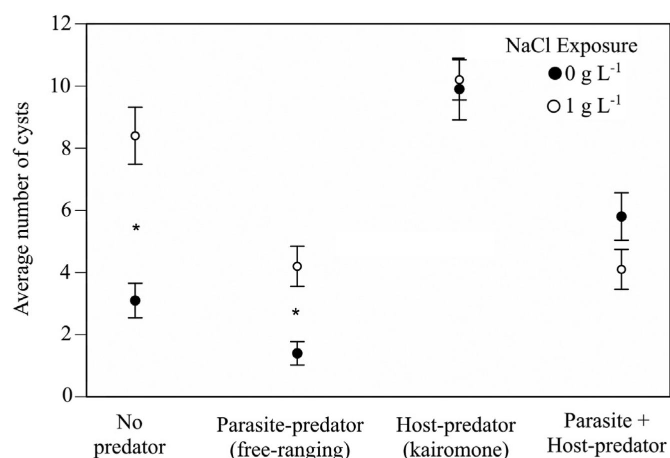

Fig. 4. Number of cercariae encysted in tadpoles (average ± SE) that had been previously exposed to 0 and $1\,g\,L^{-1}$ NaCl, across no predator, free-ranging parasite-predator (damselfly), non-lethal host-predator (dragonfly kairomone), and combination of both free-ranging parasite-predator and dragonfly kairomone treatments. Treatments with asterisks indicate a significant effect of NaCl on parasite infection ($p < 0.05$).

infection, but this increase was not associated with changes in tadpole activity. We exposed tadpoles to NaCl for only 24 h, thus it is possible that the lack of altered activity may be due to the relatively short exposure-period used in this study. An alternative mechanism for why NaCl increased tadpole susceptibility to echinostomes may be that saline environments impair kidney function in tadpole larvae which is the same organ that echinostome cercariae target (Chinathamby et al., 2006). Salinized environments are also osmotically stressful for larval amphibians and have been shown to significantly reduce lymphocyte counts at acute exposure periods of 2 h in *Notophthalmus viridescens* (Bennett and Johnson, 1973). Elevated salinity concentrations can also increase corticosterone levels in amphibians leading to reductions in immunocompetence (Gomez-Mestre et al., 2004). Ultimately, more studies are needed to evaluate the causal mechanisms for increased echinostome infection in amphibians acutely exposed to NaCl.

While NaCl exposure caused an increase in parasite infection in Experiment 1, Experiment 2 demonstrated that the effects of NaCl on host-parasite outcomes were modified by both parasite and host-predators. In Experiment 2, we confirmed our findings from Experiment 1 that exposure to NaCl ($1\,g\,L^{-1}$) caused an increase in parasite infection in the absence of either parasite – or – host - predators. Similar to tadpoles in the predator-free treatment, NaCl exposure also increased parasite infection in the presence of a free-ranging parasite-predator, but had no influence on parasite infection when tadpoles were exposed to dragonfly kairomone, or a combination of both free-ranging parasite-predator and dragonfly kairomone.

Overall, tadpoles in the free-ranging parasite-predator treatment exhibited a 51% reduction in parasite infection compared to tadpoles not exposed to either parasite or host-predators. Past work has found that damselflies reduce cercariae abundance by more than 60% (Orlofske et al., 2012, 2014). Similar to these studies, we also found that damselflies used in this study reduced cercariae abundance by 73% in our predation assays (Appendix). By reducing the number of cercariae, and thus the number of cercariae capable of encysting within the tadpole host, damselfly mitigated some of the negative effects of NaCl on infection risk. While most studies examining the role of predation in contaminant-host-parasite interactions have focused largely on the influence of host-predators (Kerby et al., 2011), it is clear that parasite-predators can also alter infection outcomes in the presence of contaminants as well via density-mediated effects. In addition to density-mediated effects on infection outcomes, we also found that damselflies reduced tadpole activity by an average of 53% compared to tadpoles not exposed to either host or parasite-predator. This reduction in tadpole activity was similar to findings by Orlofske et al. (2014), which demonstrated that the presence of free-ranging damselflies reduced tadpole activity by 13%. These findings suggest that tadpoles may not be able to interpret non-predatory invertebrates from those that are predatory (Ferrari and Chivers, 2011). Despite the reduction in tadpole activity, our results show that any effect of our free-ranging parasite-predator reducing tadpole anti-parasite behavior seemed to have been offset by the consumptive effects of the parasite-predator reducing cercariae abundance. Indeed, past works have shown that cercariae predators are highly influential in amphibian-trematode infection outcomes within wild amphibian populations, both increasing and decreasing infection outcomes (Rohr et al., 2015). Our work further corroborates the important role that parasite-predators play in influencing infection outcomes.

Contrary to the free-ranging parasite-predator and no-predator treatments, tadpole exposure to NaCl did not alter parasite infection in the presence of the dragonfly kairomone. However, in the absence of NaCl, tadpoles exposed to dragonfly kairomones were more heavily infected (a 219% increase in infection) relative to

tadpoles in the no predator treatment. When exposed to free-swimming cercariae, tadpoles display increased activity, which can reduce cercariae penetration, and thus overall parasite infection (Daly and Johnson, 2011; Taylor et al., 2004). In our study, we found that exposure to dragonfly kairomone reduced tadpole activity by 75% relative to tadpoles in the no-predator treatment, which is likely driving the increase in parasite infection. Similarly, past studies that have empirically reduced tadpole activity using anesthesia and exposure to predators (e.g. fish, damselfly and dragonfly nymphs) have also found that reduced activity is associated with increased parasite infection (Thiemann and Wassersug, 2000; Daly and Johnson, 2011; Orlofske et al., 2014). This finding underscores the importance of considering biotic stressors when examining the impacts of contaminants on host-parasite interactions and infection outcomes.

Similar to the dragonfly kairomone treatment, NaCl exposure did not influence parasite infection in tadpoles exposed simultaneously to both the free-ranging parasite-predator and dragonfly kairomone. Indeed, our findings suggest an interaction between the density-mediated effect of the free-ranging parasite-predator (cercariae consumption) and trait-mediated effect of the dragonfly kairomone (reduced activity) in the combined parasite and host-predator treatment, leading to an intermediate infection intensity relative to either of the parasite or host-predator treatments on their own. Tadpoles exposed to both parasite and host-predator were also less infected relative to tadpoles exposed only to dragonfly kairomone. Ultimately, while the presence of both predators lowered tadpole activity, heightening the chance for parasite infection, density-mediated effects of the free-ranging parasite-predator (reduced cercariae abundance due to damselfly consumption) seemed to have negated these effects, leading to an intermediate infection intensity. This finding further highlights the importance of including multiple predator-mediated effects when examining the impacts of contaminants on host-parasite interactions and infection outcomes.

Overall, our research highlights the importance of examining the effects of contaminants on host-parasite interactions within a more realistic framework by including other members of the ecological community. Collectively, this research suggests that overlooking ecological complexity may lead to incomplete conclusions of the effects that environmental contaminants, such as NaCl, may have on host-parasite outcomes. Despite these findings, our overall understanding of how contaminant-mediated effects on amphibian-parasite outcomes may be altered by other members of the community is limited (Kerby et al., 2011). By further integrating experimental approaches from both ecotoxicology and disease ecology, researchers will be better able to predict how environmental contaminants will affect disease outcomes in natural systems.

Future considerations: We demonstrate that NaCl can alter host-parasite outcomes in an amphibian-trematode system, however, our study was designed to isolate the effects of acute NaCl exposure (24 h) on only the amphibian hosts. Acute exposures are standard in toxicological assays using amphibian species (Kerby et al., 2010), but further studies examining the effects of more prolonged NaCl exposure on amphibian hosts will be necessary to understand how chronic NaCl exposure may affect host-parasite interactions in this system. Our study also only examined the effects of NaCl on the host and not the responses of predators and parasites. This is an important next step as the effects of NaCl on disease outcome can also be influenced by how predators or parasites respond to NaCl. Finally, amphibian species can vary widely in their tolerance to NaCl (Karraker et al. 2008; Collins and Russell, 2009; Hua and Pierce, 2013). These interspecific differences in tolerance to NaCl may influence parasite infection outcomes. For instance, amphibian species that are more tolerant of NaCl, such as American toads (*Anaxyrus americanus*; Collins and Russell, 2009), may be less susceptible to parasites following exposure to NaCl compared to less NaCl-tolerant species, such as *L. sylvaticus* (Collins and Russell, 2009). Further work examining the role that species-level tolerance to NaCl may play in altering infection outcomes will ultimately allow for predictions as to which amphibian species will be most affected by salinization and parasite infections in nature. To sum, as secondary salinization in freshwater systems continues to increase over time, predicting the impacts of salinization on trematode infection will be valuable in assessing disease risk in amphibian populations.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.envpol.2018.01.060.

Table 1A
GLM output showing intercept estimates for parasite infection in treatment groups against our control group (tadpoles not exposed to NaCl). P-values in bold represent significant differences in respective treatments when compared with the control.

NaCl Treatment	B	Std. Error	95% Wald CI		Wald χ^2	p-value
			Lower	Upper		
1 g L^{-1}	0.520	0.09	−1.631	0.349	35.636	**0.001**
2 g L^{-1}	0.488	0.09	−2.602	0.319	31.924	**0.001**
3 g L^{-1}	0.674	0.08	−1.965	0.511	65.095	**0.001**

Table 2A
GLM output showing intercept estimates for tadpole movement in treatment groups against our control group (tadpoles not exposed to either NaCl or predators). P-values in bold represent significant differences in respective treatments when compared with the control.

Treatment	B	Std. Error	95% Wald CI		Wald χ^2	p-value
			Lower	Upper		
NS*PP	−0.713	0.47	−1.631	0.206	2.313	0.128
NS*HP	−1.642	0.49	−2.602	−0.682	11.247	**0.001**
NS*HPPP	−1.036	0.47	−1.965	−0.107	4.779	**0.029**
S*NP	−0.649	0.47	−1.566	0.268	1.925	0.165
S*PP	−1.517	0.49	−2.469	−0.565	9.758	**0.002**
S*HP	−1.517	0.49	−2.469	−0.565	9.758	**0.002**
S*HPPP	−1.193	0.48	−2.129	−0.258	6.253	**0.012**

NS (No Salt) = Tadpoles not exposed to NaCl.
S (Salt) = Tadpoles exposed to NaCl.
NP (No Predators) = Tadpoles not exposed to either parasite or host predators.
PP (Parasite Predator) = Tadpoles exposed to only parasite predators.
HP (Host Predator) = Tadpoles exposed only to host predators.
HPPP (Host Predator and Parasite Predator) = Tadpoles exposed to both host predator and parasite predator.

Table 3A
GLM output showing intercept estimates for parasite infection in treatment groups against our control group (tadpoles not exposed to either NaCl or predators). P-values in bold represent significant differences in respective treatments when compared with the control.

Treatment	B	Std. Error	95% Wald CI		Wald χ^2	p-value
			Lower	Upper		
NS*PP	0.997	0.21	0.585	1.409	6.094	**0.001**
NS*HP	−0.795	0.32	−1.426	−0.164	1.645	**0.014**
NS*HPPP	0.304	0.24	−0.160	0.768	31.829	0.200
S*NP	1.161	0.21	0.758	1.565	33.723	**0.001**
S*PP	1.191	0.21	0.789	1.593	7.928	**0.001**
S*HP	0.626	0.22	0.190	1.063	1.380	**0.005**
S*HPPP	0.280	0.24	−0.187	0.746	22.500	0.240

NS (No Salt) = Tadpoles not exposed to NaCl.
S (Salt) = Tadpoles exposed to NaCl.
NP (No Predators) = Tadpoles not exposed to either parasite or host predators.
PP (Parasite Predator) = Tadpoles exposed to only parasite predators.
HP (Host Predator) = Tadpoles exposed only to host predators.
HPPP (Host Predator and Parasite Predator) = Tadpoles exposed to both host predator and parasite predator.

Table 1B
Planned contrasts (sequential-Bonferroni) showing mean differences in tadpole activity between treatments and associated p-values. Significant differences between treatments are shown in bold.

	NS*NP	NS*PP	NS*HP	NS*HPPP	S*NP	S*PP	S*HP	S*HPPP
NS*NP		7.9 (0.163)	12.50 (**0.016**)	10.00 (0.064)	7.4 (0.197)	12.1 (**0.020**)	12.1 (**0.020**)	10.8 (**0.042**)
NS*PP	7.9 (0.163)		4.6 (0.098)	2.1 (0.509)	0.5 (0.893)	4.2 (0.138)	4.2 (0.138)	2.9 (0.339)
NS*HP	12.50 (**0.016**)	4.6 (0.098)		2.5 (0.253)	5.1 (0.082)	0.4 (0.808)	0.4 (0.808)	1.7 (0.388)
NS*HPPP	10.00 (0.064)	2.1 (0.509)	2.5 (0.253)		2.6 (0.432)	2.1 (0.351)	2.1 (0.351)	0.8 (0.749)
S*NP	7.4 (0.197)	0.5 (0.893)	5.1 (0.082)	2.6 (0.432)		4.7 (0.114)	4.7 (1.140)	3.4 (0.283)
S*PP	12.1 (**0.020**)	4.2 (0.138)	0.4 (0.808)	2.1 (0.351)	4.7 (0.114)		0.0 (1.000)	0.1 (1.000)
S*HP	12.10 (**0.020**)	4.2 (0.138)	0.4 (0.808)	2.1 (0.351)	4.7 (1.140)	0.0 (1.000)		1.30 (0.525)
S*HPPP	10.8 (**0.042**)	2.9 (0.339)	1.7 (0.388)	0.8 (0.749)	3.4 (0.283)	0.1 (1.000)	1.30 (0.525)	

NS (No Salt) = Tadpoles not exposed to NaCl.
S (Salt) = Tadpoles exposed to 1 g L^{-1} NaCl for 24 h.
NP (No Predators) = Tadpoles not exposed to either parasite or host predators.
PP (Parasite Predator) = Tadpoles exposed to only parasite predators.
HP (Host Predator) = Tadpoles exposed only to host predators.
HPPP (Host Predator and Parasite Predator) = Tadpoles exposed to both host predator and parasite predator.

Table 2B
Planned contrasts (sequential-Bonferroni) showing mean differences in parasite infection between treatments and associated p-values. Significant differences between treatments are shown in bold.

	NS*NP	NS*PP	NS*HP	NS*HPPP	S*NP	S*PP	S*HP	S*HPPP
NS*NP		1.7 (0.113)	6.8 (**0.001**)	2.7 (**0.046**)	5.3 (**0.001**)	1.1 (1.000)	7.1 (**0.001**)	1.0 (1.000)
NS*PP	1.7 (0.113)		8.5 (**0.001**)	4.4 (**0.001**)	7.0 (**0.001**)	2.8 (**0.003**)	8.8 (**0.001**)	2.7 (**0.004**)
NS*HP	6.8 (**0.001**)	6.8 (**0.001**)		4.1 (**0.013**)	1.5 (1.000)	5.7 (**0.001**)	0.3 (1.000)	5.8 (**0.001**)
NS*HPPP	2.7 (**0.046**)	2.7 (**0.046**)	4.1 (**0.013**)		2.6 (0.262)	1.6 (0.767)	4.4 (**0.007**)	1.7 (1.000)
S*NP	5.3 (**0.001**)	5.3 (**0.001**)	1.5 (1.000)	2.6 (0.262)		4.2 (**0.003**)	1.8 (1.000)	4.3 (**0.002**)
S*PP	1.1 (1.000)	1.1 (1.000)	5.7 (**0.001**)	1.6 (0.767)	4.2 (**0.003**)		6.0 (**0.001**)	0.1 (1.000)
S*HP	7.1 (**0.001**)	7.1 (**0.001**)	0.3 (1.000)	4.4 (**0.007**)	1.8 (1.000)	6.0 (**0.001**)		6.1 (**0.001**)
S*HPPP	1.0 (1.000)	1.0 (1.000)	5.8 (**0.001**)	1.7 (1.000)	4.3 (**0.002**)	0.1 (1.000)	6.1 (**0.001**)	

NS (No Salt) = Tadpoles not exposed to NaCl.
S (Salt) = Tadpoles exposed to 1 g L^{-1} NaCl for 24 h.
NP (No Predators) = Tadpoles not exposed to either parasite or host predators.
PP (Parasite Predator) = Tadpoles exposed to only parasite predators.
HP (Host Predator) = Tadpoles exposed only to host predators.
HPPP (Host Predator and Parasite Predator) = Tadpoles exposed to both host predator and parasite predator.

Appendix C. Methods and results for cercariae predation assays using damselfly nymphs (*Enellagma* sp.)

Methods

To examine the degree to which damselfly nymphs (*Enellagma* sp.) can reduce echinostome cercariae abundance, we conducted a lab predation assay on 20 June 2016. We obtained cercariae for this assay by placing three infected snails in separate 50 mL Falcon tubes filled with 35 mL of aged well-water and exposed them to a heat lamp for 1 h to induce cercariae shedding. For the assay, we placed damselfly nymphs individually into 20 containers filled with 80 mL of well-water. We then pipetted 10 cercariae from a mixture of the three snails that were shed into each of the 20 containers. We removed the damselfly nymph and recovered and recorded the remaining number of cercariae using a dissecting scope 1 h after adding cercariae to each unit. To determine how well our methods worked for recovering and recording remaining cercariae we also set up 20 control containers, also filled 80 mL of well-water. These containers contained no damselfly nymphs and cercariae abundance in each unit was recorded after 1 h.

Results

We successfully recovered 100% of the cercariae that were added to our damselfly-free units, showing the efficacy of our methodology for recovering cercariae. Within our damselfly treatments we found that after 1 h, damselfly nymphs reduced cercariae abundance by an average of 73%. Damselfly consumed a proportion of the total cercariae in all 20 experimental units.

Table 1C
Pairwise comparisons showing differences in tadpole activity between predator treatments from the scan sampling assay.

Predator comparison	Mean Diff	Std. Error	p-value
No Predator * Damselfly	0.67	0.401	0.097
No Predator *Kairomone	1.13	0.386	0.003
No Predator * Both Predators	1.06	0.388	0.007
Damselfly * Kairomone	0.46	0.363	0.203
Damselfly * Both Predators	0.39	0.366	0.286
Kairomone * Both Predators	0.07	0.349	0.836

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