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Effects of different roadway deicing salts on host-parasite interactions: The importance of salt type☆

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ABSTRACT

The application of roadway deicing salts is increasing the salinity of freshwater systems. Increased salinization from salts, such as NaCl, $CaCl_2$ and $MgCl_2$, can have direct, negative impacts on freshwater organisms at concentrations found in nature. Yet, our understanding of how these salts can indirectly impact freshwater organisms by altering important ecological interactions, such as those between hosts and their parasites, is limited. Using a larval amphibian and infectious free-living helminth (i.e. trematode) model, we examined whether exposure to environmentally relevant concentrations of NaCl, $CaCl_2$ and $MgCl_2$ 1) influence trematode mortality; 2) alter amphibian-trematode interactions; and 3) alter larval amphibian activity (a behavior associated with parasite avoidance). We found that exposure to $CaCl_2$ greatly reduced trematode survival across all Cl^- concentrations (230, 500, 860 and 1000 mg Cl^- L^{-1}) while NaCl and $MgCl_2$ had no effect. When both host and parasites were exposed to the salts, exposure to NaCl, but not $MgCl_2$ or $CaCl_2$, increased infection. The lack of effect of $CaCl_2$ on infection was likely driven by $CaCl_2$ reducing trematode survival. Exposure to NaCl increased infection at 500 mg Cl^- L^{-1}, but not 230 or 860 mg Cl^- L^{-1}. Increased infection was not due to salt exposure altering tadpole behavior. Our results suggest that NaCl can negatively impact amphibian populations indirectly by increasing trematode infections in tadpole hosts.

1. Introduction

Anthropogenic activities are threatening the health and stability of freshwater ecosystems worldwide (Dudgeon et al., 2006; Reid et al., 2019). One such threat is the increased accumulation of salts in freshwater systems (i.e. secondary salinization) (Cañedo-Argüelles et al., 2016; Dugan et al., 2017; Schuler et al., 2018). Different from primary, or natural salinization, secondary salinization can be the direct result of anthropogenic activities. For example, common sources of secondary salinization include runoff from irrigation practices in agriculture or from the use of deicing salts on roadways (Hill and Sadowski, 2016). The use of deicing salts is of particular concern, as their use has led to increased salinization of freshwater systems across North America (Dugan et al., 2017; Evans and Frick, 2001; Findlay and Kelly, 2011; Hintz and Relyea, 2019; Kaushal et al., 2005). Deicing salts, such as sodium chloride (NaCl), magnesium chloride ($MgCl_2$) and calcium chloride ($CaCl_2$) are the most commonly used, with NaCl being the most common (Evans and Frick, 2001).

The direct impacts of salinization from these salts on freshwater organisms are numerous. For example, salinization can negatively impact the physiology (Burraco and Gomez-Mestre, 2016), growth/development (Hintz and Relyea, 2017; Hua and Pierce, 2013; Luz et al., 2008), reproduction (Ghazy et al., 2009), behavior (Denoël et al., 2010) and even immune functions (Birrer et al., 2012; Mangahas et al., 2019) of freshwater organisms (Hintz and Relyea, 2019). In addition to these direct effects, secondary salinization can also indirectly influence freshwater organisms by modifying species interactions, yet our understanding of salt modifying species interactions is limited (Hintz and Relyea, 2019). Evaluating the indirect consequences of secondary salinization on ecological interactions will become increasingly important as anthropogenic activities continue to intensify the degree of salinization across freshwater systems (Dugan et al., 2017).

Infectious diseases in wildlife populations have increased over the past several decades (Cunningham et al., 2017; Daszak et al.,

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2000). While these increases in disease are thought to be caused by several factors, exposure to environmental pollutants have been hypothesized to be one contributing factor (Acevedo-Whitehouse and Duffus, 2009). Indeed, several studies suggest that increased levels of salinization can alter host-parasite interactions, modifying disease outcomes (Buss and Hua, 2018; Hall et al., 2020, 2013; Milotic et al., 2017; Stockwell et al., 2015; Studer and Poulin, 2012). Salt-mediated changes to host-parasite outcomes can occur via effects on either the host or parasite. For example, elevated levels of salinity can compromise the immune function of freshwater hosts (Birrer et al., 2012; Mangahas et al., 2019), which can increase host susceptibility to parasitic infections (Buss and Hua, 2018). Alternatively, exposure to elevated salinity can suppress behavioral traits that allow hosts to avoid parasitic infection (Milotic et al., 2017). For parasites, exposure to elevated salinities can reduce the motility of free-living infectious stages of the parasite, lowering the risk of infection to hosts, as is the case with the amphibian chytrid fungus, *Batrachochytrium dendrobatidis* (Stockwell et al., 2015). Alternatively, elevated salinities can reduce the survival of free-living infectious stages, limiting host infection risk through decreases in parasite abundance (Pietrock and Marcogliese, 2003). Given these potential outcomes, it will be critical to further examine how elevated salinities influence host-parasite interactions via effects on both hosts and their parasites.

Despite the increasing use of $MgCl_2$ and $CaCl_2$ as alternative roadway deicers, most research on the effects of elevated salinity on host-parasite interactions has focused on the effects of NaCl (Buss and Hua, 2018; Hall et al., 2013; Merrick and Searle, 2019; Milotic et al., 2017). This is of concern, as different forms of salt (i.e. NaCl, $MgCl_2$ and $CaCl_2$) can differentially impact freshwater organisms. For example, Hintz and Relyea (2017) found that $CaCl_2$ and NaCl, but not $MgCl_2$, reduce the growth of rainbow trout (Oncorhynchus mykiss) even when Cl-concentrations were held constant between salt types. Thus, roadway deicing salts may differentially impact host-parasite interactions in a similar fashion. By evaluating the effects of the most commonly used roadway deicing salts on host-parasite interactions, we can better manage the degree to which salinization impacts host-parasite outcomes.

Using an amphibian-trematode model, we aimed to determine whether exposure to environmentally relevant concentrations of NaCl, $CaCl_2$ and $MgCl_2$ 1) influences mortality in parasites; 2) alters host-parasite interactions; and 3) alters the activity of hosts (a trait associated with parasite avoidance behaviors).

2. Methods

2.1. Host-parasite model

The amphibian-trematode model is an excellent model for testing the effects of salt exposure on disease outcome for several reasons. For example, amphibian populations are declining worldwide (Wake and Vredenburg, 2008) and it has been hypothesized that these declines may be driven in part by environmental contaminants and disease (Blaustein et al., 2011; Daszak et al., 2000; Hayes et al., 2010). Thus, understanding how the most commonly applied roadway deicing agents impact disease outcomes for amphibian taxa could prove useful to their management and conservation. In addition, while the free-living cercaria life stage of a number of trematode species appear to be intolerant of exposure to some environmental contaminants (e.g. pesticides and metals; Hua et al., 2016; Morley et al., 2002), cercariae across a number of trematode species appear to be relatively tolerant of NaCl exposure (Donnelly et al., 1984; Milotic et al., 2020; Shostak, 1993; Venable et al., 2000). For example, Donnelly et al. (1984) found no impact of NaCl exposure on the survival of three schistosome species until concentrations exceeded 5250 mg L^{-1} NaCl. Similarly, Milotic et al. (2020) found no decline in the survival of cercariae of *Ribeiroia ondatrae*, *Echinostoma* sp., or a strigeid-type trematode at concentrations of 160 - 960 mg L^{-1} NaCl (Milotic et al., 2020). Cercariae of the trematodes *Bolbophorus confusus* and *Petasiger nitidus* show similar tolerances, with *B. confusus* cercariae showing no decline in survival at concentrations of 1, 250 and 2500 mg L^{-1} NaCl (Venable et al., 2000) and *P. nitidus* surviving at concentrations of up to 2900 mg L^{-1} NaCl (Shostak, 1993). However, the effects of salts other than NaCl on cercaria survival, such as $MgCl_2$ and $CaCl_2$, are unknown.

Wood frog (*Lithobates sylvaticus*) tadpoles were our model host. Wood frogs are one of the most widely distributed species of anurans in North America (Conant and Collins, 1998) and inhabit salinized wetlands (Brady, 2013). With regard to salinization and wood frog-trematode dynamics, past research has shown that both acute exposure of larvae to NaCl (1 d) at concentrations > 1000 mg L^{-1} (Buss and Hua, 2018) and chronic exposure of larvae to NaCl (12 d) at concentrations of 600 and 1050 mg L^{-1} (Milotic et al., 2017) increase the infection success of trematodes to larval hosts. When in the presence of trematode cercariae, tadpoles can display highly effective parasite avoidance behaviors, such as increases in activity (Koprivnikar et al., 2006). Previous work suggests that exposure of wood frogs to NaCl limits their activity levels (Sanzo and Hecnar, 2006), and was suggested to be the main mechanism behind increased trematode infections of NaCl-exposed wood frogs seen by Milotic et al. (2017). However, whether exposure to $MgCl_2$ or $CaCl_2$ similarly impacts wood frog behavior, and subsequently impacts the infection success of trematodes to wood frog hosts, is unknown. Moreover, NaCl, $MgCl_2$ and $CaCl_2$ can differentially impact the survival of wood frog larvae (Harless et al., 2011). This differential tolerance to the three salts suggests that exposure may also differentially impact the infection success of trematodes to wood frog larvae. This is important as it could inform the application of salts near breeding habitats of wood frogs if one salt type were to impact the success of trematode infections to hosts less than the others.

Digenetic trematodes within the family Echinostomatidae were our model parasite. Trematodes within this family utilize a multi-host lifecycle, with amphibians serving as the second intermediate host (Johnson and McKenzie, 2009). Specifically, we worked with the cercarial life stage of echinostomes. The cercarial life stage of these parasites is free-living, and actively seeks out larval amphibian hosts in the environment. If successful in infecting a tadpole, the cercaria forms a metacercarial cyst within the tadpole's kidneys (Fried et al., 1998; Kanev et al., 2000). Infection by these parasites can induce edema through renal failure (Lannoo and Lannoo, 1998), increase time to metamorphosis and growth (Orlofske et al., 2017), and can cause mortality at relatively low levels of infection ($\leq$51 metacercarial cysts) at early developmental stages (Schotthoefer et al., 2003). Given that echinostome cercariae are free-living in the aquatic environment, they are susceptible to toxicant exposure (Sures et al., 2017). Importantly, echinostome cercariae are most infectious to tadpole hosts within the first 8 h of leaving their gastropod hosts, with infectivity declining thereafter (Fried and Graczyk, 1997). As such, any changes to cercariae survival due to toxicant exposure during this period could limit the infection of wood frogs by echinostomes. Regarding road salt exposure and echinostome cercariae, Milotic et al. (2020) found no decline in *Echinostoma* sp. cercariae survival when cercariae were exposed to NaCl across a broad range of environmentally relevant concentrations (160–960 mg NaCl L^{-1}). However, whether exposure of echinostome cercariae to $CaCl_2$ or $MgCl_2$ impacts their survival is unknown. Thus, it is important to consider how these other forms of road salts may impact echinostome survival, as reduced survival,

particularly during their most infectious period, could have direct implications for amphibian-trematode infection dynamics within salinized systems.

2.2. Animal collection and husbandry

We collected 10 partial wood frog egg masses on April 19, 2019 from a wetland located in Rensselaer County, NY (42.722391, −73.606812). Following collection, we transported egg masses to the Rensselaer Aquatic Laboratory (Troy, NY). We held egg masses in 120-L plastic pools filled with 100 L of water (2 partial masses/pool) and covered each pool with 70% shade cloth. Once the eggs hatched, we fed the tadpoles rabbit chow *ad libitum* and conducted weekly water changes until the start of the experiment.

To obtain cercariae for our experiments, we collected >200 and 86 ramshorn snails (*Helisoma trivolvis*) from a permanent wetland located in Rensselaer County, NY (42.7702550, −73.6446519) on 24 June and July 29, 2019, respectively. Because these snails can serve as hosts for several species of parasites, we screened all snails to identify individuals infected with trematodes belonging to the family Echinostomatidae (i.e. echinostomes), which includes species in the Echinostoma and Echinoparyphium genera. To confirm that a snail was infected with echinostomes, we individually exposed a subset of tadpoles to cercariae. After 24 h of exposure, we euthanized the tadpoles in a 200 mg L^{-} MS-222 solution and then dissected out the kidneys of each tadpole to confirm the presence or absence of metacercarial cysts, which are indicative of infection by echinostomes. We additionally assessed that cercariae were from the family Echinostomatidae through morphological observations (Kanev et al., 2000; Schell, 1985). We held snails with confirmed echinostome infection in 15 L tubs filled with 10 L of water for 24 h until the start of their respective experiments. Snails were not fed during this time.

2.3. Effect of salts on cercariae survival

To evaluate the direct, lethal effect of salt on cercariae, we conducted a time-to-death experiment on July 30, 2019. In this experiment, we exposed cercariae to three salt types (NaCl, $MgCl_2$ and $CaCl_2$) at four Cl^{-} concentrations (230, 500, 860 and 1000 mg Cl^{-} L^{-1}) and a water control (25 mg L^{-1} Cl^{-} control). Thus, there was a total of 13 treatments. Cl^{-} concentrations were held constant between salt types to evaluate how the anions of each salt type impact amphibian-trematode dynamics. To make the $MgCl_2$ and $CaCl_2$ salt solutions used throughout all of our experiments we used Safe Step® salts (Compass Minerals, Kansas, USA; 100% $MgCl_2$ and $CaCl_2$, respectively). To make the NaCl Salt solutions we used Morton® Safe-T Salt® (Morton Company, Illinois, USA; > 99% NaCl). For nominal versus actual concentrations see Table 1.

The concentrations of Cl^{-} used here and throughout our experiments represent environmentally relevant concentrations for wetlands affected by secondary salinization (Kaushal et al., 2005; Sanzo and Hecnar, 2006). For example, a review of wetland salinization due to road salt contamination found Cl^{-} concentrations in wetlands ranging from 10 mg Cl^{-} L^{-1} up to 13,500 mg Cl^{-} L^{-1} (Hintz and Relyea, 2019). The concentrations of 230 and 860 mg Cl^{-} L^{-1} represent values for chronic and acute exposure limits of Cl^{-} for freshwater organisms established by the United States EPA, respectively (US EPA, n.d.). However, the 230 and 860 mg Cl^{-} L^{-1} concentrations used here are above the chronic (160 mg Cl^{-} L^{-1}) and acute (640 mg Cl^{-} L^{-1}) chloride thresholds set by the Canadian government (Government of Canada, 2004).

To obtain cercariae, we shed cercariae from 10 snails on July 30, 2019 by placing them into individual 50 mL centrifuge tubes filled with 30 mL of water. The tubes were placed under a heat lamp for 1 h. Following cercarial emergence from the snails, we used a dissecting microscope to pipette a single cercaria into experimental units. Experimental units were individual wells within seven 24-well cell culture plates with 2 mL of treatment water added to each well. We randomly assigned treatments across these seven plates. We replicated the 13 treatments 12 times, for a total of 156 experimental units. To prevent cross-contamination of wells, we used separate glass pipettes to add cercariae to each respective treatment.

Following the addition of cercariae to the wells, we checked for mortality of cercariae every hour for 14 h. We conducted checks by scanning for movement of cercariae within each well using a dissecting microscope. If cercariae were not moving, we used a glass pipette to direct a stream of water at the cercariae (i.e., mechanical stimulation) to stimulate movement. Cercariae were considered dead if they did not respond to mechanical stimulation, a common criterion for cercariae mortality assays (Buss et al., 2019; Hua et al., 2016; Rohr et al., 2008). We ended the experiment at hour 14, at which point all cercariae had died. Mortality of echinostome cercariae in the controls at this hour is not unusual as the typical lifespan of echinostomes is 6–24 h after leaving their snail hosts (Hua et al., 2016; Milotic et al., 2019). Further, echinostome cercariae are most infectious within 8 h of being shed from their snail hosts (Fried and Graczyk, 1997). Thus, our experimental design allowed us to evaluate the impact of roadway deicing salts on cercariae across the period of highest relevance for their interactions with tadpole hosts.

2.4. Effect of salts on disease outcomes

To evaluate the effects of salt exposure on host-parasite interactions, we simultaneously exposed wood frog tadpoles and

Table 1
Nominal and actual Cl^{-} concentrations (230, 500, 860 and 1000 mg Cl^{-} L^{-1}) across three salt types (NaCl, $MgCl_2$ and $CaCl_2$) that echinostome cercariae were exposed to in Experiment 1 (*Effect of salts on cercariae survival*).

Salt type	Nominal Cl^{-} concentration (mg L^{-1})	Actual Cl^{-} concentration (mg L^{-1})
MgCl2	230	255
MgCl2	500	440.6
MgCl2	860	781.2
MgCl2	1000	933.6
NaCl	230	260.8
NaCl	500	454.2
NaCl	860	806.7
NaCl	1000	956.3
CaCl2	230	262.6
CaCl2	500	426.2
CaCl2	860	799.4
CaCl2	1000	942.2

echinostome cercariae to three types of roadway deicing salts: NaCl, $CaCl_2$ and $MgCl_2$ at three concentrations of Cl^-: 230, 500 and 860 mg Cl^- L^{-1}. We also included a water control (25 mg Cl^- L^{-1}). For nominal versus actual concentrations see Table 2.

On June 12, 2019, we haphazardly selected 24 tadpoles from each of our five common-garden pools, for a total of 120 tadpoles (Gosner stages: 30–34; average Gosner stage ± SE: 32.25 ± 0.11). We allowed tadpoles to acclimate in lab conditions (20 °C and 12:12 light cycle) for 24 h before the start of the experiment. After the acclimation period, we haphazardly placed individual tadpoles into 1 L plastic experimental units filled with 800 mL of treatment or control water randomized across three shelving units at the same height (day 0 of the experiment). The 10 treatments were replicated 12 times for a total of 120 experimental units.

We fed tadpoles a Tetrafin slurry every day (4% of initial body mass) and conducted a water change on day 4. To determine the amount of food to add, we weighed a separate group of 15 tadpoles held in similar conditions (average starting mass ± SE = 126.5 ± 5.5 mg). We used this separate group to avoid stressing or injuring the experimental animals.

On 25 June (day 7 of the experiment), we shed cercariae from 12 infected snails collected on 24 June, following the methodologies mentioned above. Follow cercarial emergence from the snails, we pipetted 30 cercariae into 120 separate 10-mL scintillation vials filled with 2 mL of water. We poured the contents of the scintillation vials into each of the 120 experimental units containing wood frog hosts, rinsing the vials three times to assure the addition of all 30 cercariae to each experimental unit. Exposure of larval wood frogs to this number of echinostome cercariae is environmentally-relevant per surveys of echinostome infections in wild populations (Woodhams et al., 2000). To maximize genetic variation of parasites going into each experimental unit, we added a random combination of cercariae from each of the 12 snails into each experimental unit. Given that echinostome cercariae encyst in the kidneys of tadpoles within 24 h (Rohr et al., 2008), we terminated the experiment 24 h after the addition of the cercariae. We euthanized all animals in a 200 mg L^{-1} MS-222 solution and then individually preserved them in a 10% formalin solution. We determined the developmental stage (Gosner, 1960) and mass of all individuals. To quantify trematode infection, we removed the kidneys of each tadpole and counted the number of metacercarial cysts between glass slides using a dissecting microscope.

2.5. Effect of salts on tadpole activity

Tadpoles commonly display increased activity in the presence of cercariae to limit infections (Daly and Johnson, 2011; Koprivnikar et al., 2006; Milotic et al., 2017). Exposure to NaCl can reduce the ability of tadpoles to increase their activity in the presence of cercariae, leading to increased infections (Milotic et al., 2017). Thus, exposure to the salts used in our study could similarly increase infection by reducing tadpole activity. To understand how each of the salt treatments influenced the activity levels of tadpoles across time, we conducted three scan sampling assays (days 2, 4, 6 and 7; Relyea and Mills, 2001). Scan sampling has been shown to be capable of detecting differences in tadpole activity in response to competitors (Relyea, 2002) predators (Relyea, 2001; Schoeppner and Relyea, 2009) and trematode cercariae (Raffel et al., 2010). At each time point, observers approached the units and recorded whether tadpoles were moving or not. After observation of a unit, the observer moved to the next unit and recorded tadpole activity (yes/no) and repeated this until all 120 units had been scanned. This process was repeated 15 times, for a total of 15 observations for each tadpole/sampling assay. For each assay, we calculated activity within the respective treatments as the mean proportion of times tadpoles were active (i.e. # of times a tadpole moved/15).

To understand the effect of salts on tadpole activity in the presence of cercariae, we conducted a scan sampling assay before and immediately after adding cercariae to the experimental units on day 7. To ensure that we were measuring parasite avoidance behaviors and not activity arising from the addition of water to the units, we sham dosed each unit with parasite-free water prior to conducting scan sampling on day 7. We collected activity data using the same methods described above. Sampling assays across all time periods lasted 35 ± 5 min (average ± SE).

2.6. Statistical analyses

Cercariae survival –To evaluate the effects of salt exposure on cercarial survival, we used a One-way Analysis of Variance (ANOVA) test. Our response variable was the hour at which cercaria died following salt exposure, with Cl^- concentration, salt type and their interaction as fixed predictors, with the well plate number that cercariae were in as a covariate.

Host-parasite outcomes- To evaluate the relative effects of each salt treatment on tadpole infection, we used a Kruskall-Wallis nonparametric test, as our data did not conform to a normal distribution. Our response variable was the total number of metacercarial cysts within both tadpole kidneys with salt treatment as a fixed predictor.

Tadpole activity- To evaluate the effects of salt exposure on tadpole activity across time, we used an ANOVA. Our response variable was the proportion of time active/15 for each tadpole, with salt type, Cl^- concentration, day (2, 4 or 6) and interactions between each as fixed predictors.

To evaluate the effects of salt exposure on the ability of tadpoles to mount parasite avoidance behaviors, such as increased levels of activity, we used a repeated-measures ANOVA (rm-ANOVA). Our response variable was the proportion of time active/15 for each tadpole pre and post parasite addition. Salt type, Cl^- concentration and their interactions were coded as fixed predictors.

Table 2
Nominal and actual Cl^- concentrations (230, 500 and 860 mg Cl^- L^{-1}) across three salt types (NaCl, $MgCl_2$ and $CaCl_2$) that wood frog tadpoles and echinostome cercariae were exposed to in Experiment 2 (*effect of salts on disease outcomes*).

Salt type	Nominal Cl^- concentration (mg L^{-1})	Actual Cl^- concentration (mg L^{-1})
MgCl2	230	230.14
MgCl2	500	500.83
MgCl2	860	860.35
NaCl	230	230.47
NaCl	500	500.4
NaCl	860	860.53
CaCl2	230	230.27
CaCl2	500	500.56
CaCl2	860	859.7

3. Results

3.1. Cercariae survival

We found a significant overall effect of salt type and Cl^- concentration on cercariae survival ($F_{4, 156} = 3.342$; $p < 0.001$). There was a significant main effect of salt type ($p < 0.001$), but no significant main effect of Cl^- concentration ($p = 0.753$) and no interactive effect between salt type and Cl^- concentration ($p = 0.626$).

Sequential-Bonferroni-adjusted pairwise comparisons showed that relative to the control, cercariae exposed to $CaCl_2$ experienced 44% lower survival (i.e. average time alive; $p < 0.001$; Fig. 1). Additionally, cercariae exposed to $CaCl_2$ also experienced lower survival than individuals exposed to $MgCl_2$ (a 35% reduction; $p < 0.001$) and NaCl (a 35% reduction; $p < 0.001$). Cercariae exposed to $MgCl_2$ or NaCl did not differ in survival compared to the control ($p = 0.515$ and $p = 0.348$, respectively) nor compared to each other ($p = 1.00$). On average, cercariae exposed to $CaCl_2$ survived 4 h, with mortality beginning as early as 1 h following exposure.

3.2. Host-parasite outcomes

We found a significant overall effect of salt treatment on trematode infection intensity in wood frog hosts ($H = 17.027$, $p = 0.048$; Fig. 2). Sequential-Bonferroni-adjusted pairwise comparisons revealed that this effect was primarily driven by the 500 mg L^{-1} Cl—NaCl treatment, which increased infection intensity by 57% relative to the control ($p = 0.018$). No other pairwise comparisons were statistically significant ($p > 0.05$).

3.3. Tadpole activity – days 2, 4 and 6

We found a significant overall effect of our model on tadpole activity ($F_{29, 359} = 3.476$, $p < 0.001$; Fig. 3). We found significant main effects of salt type ($p = 0.037$), and day ($p < 0.001$), but no main effect of Cl^- concentration ($p = 0.567$). There was no significant interactive effect between salt type and day ($p = 0.245$), salt type and Cl^- concentration ($p = 0.108$), between day and Cl^- concentration ($p = 0.604$), or between salt type, Cl^- concentration and day ($p = 0.287$).

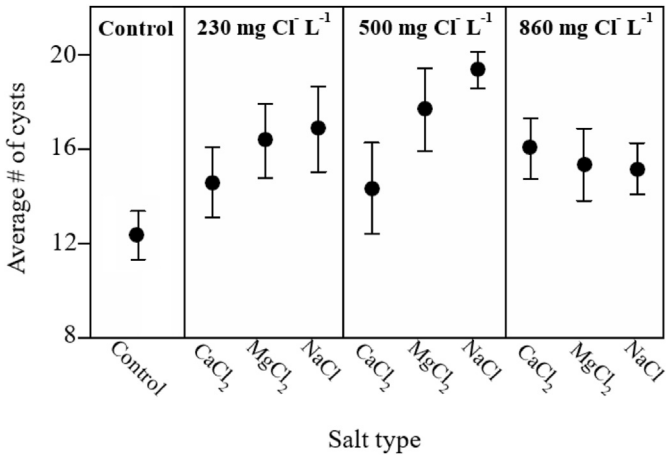

Fig. 2. Average number of metacercarial cysts within tadpole hosts (±SE) exposed to three salt types: $CaCl_2$, $MgCl_2$ and NaCl, across three Cl^- concentrations: 230, 500 and 860 mg Cl^- L^{-1} and a control (25 mg Cl^- L^{-1}). Treatments with different letters within respective panels differ significantly from one another ($p < 0.05$).

Despite the significant main effect of salt type, sequential-Bonferroni-adjusted pairwise comparisons showed no significant differences in tadpole activity between salt types ($p > 0.05$ for all). Tadpole activity differed significantly between all days, increasing with time ($p < 0.001$), except between days 4 and 6 ($p = 0.322$).

3.4. Tadpole activity – day 7, pre- and -post cercariae exposure

Using a rm-ANOVA, we examined the main effects of salt type, Cl^- concentration and their interaction on tadpole activity across two time points, once before and once after they were exposed to echinostome cercariae. The rm-ANOVA found no significant effects of salt type ($p = 0.183$), Cl^- concentration ($p = 0.209$), or their interaction ($p = 0.839$) on tadpole activity. However, there was an effect of time (i.e. cercariae exposure), with tadpoles increasing their activity on average by 13% following exposure to the cercariae

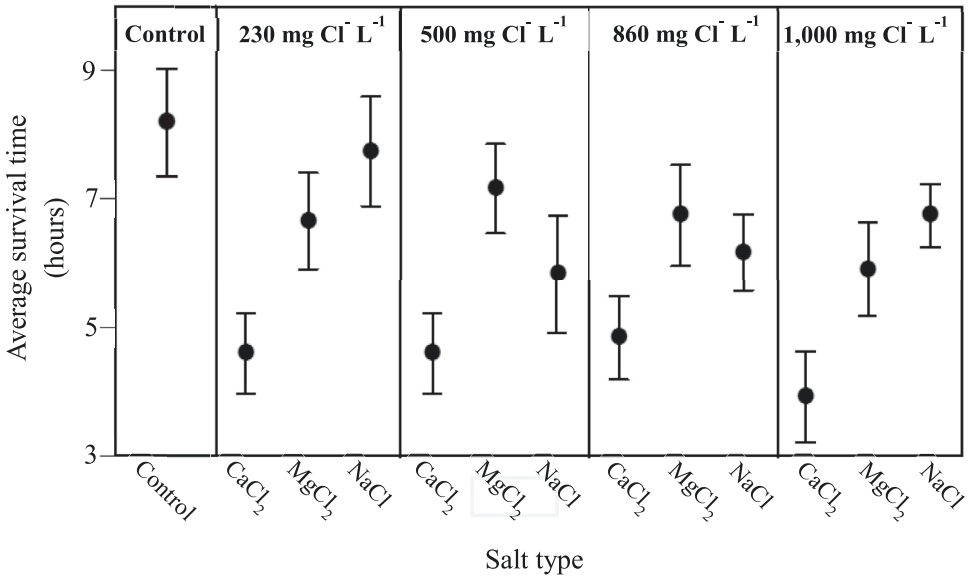

Fig. 1. Average survival time of cercariae (±SE) exposed to three salt types: $CaCl_2$, $MgCl_2$ and NaCl, across four Cl^- concentrations: 230, 500, 860 and 1000 mg Cl^- L^{-1} and a control (25 mg Cl^- L^{-1}).

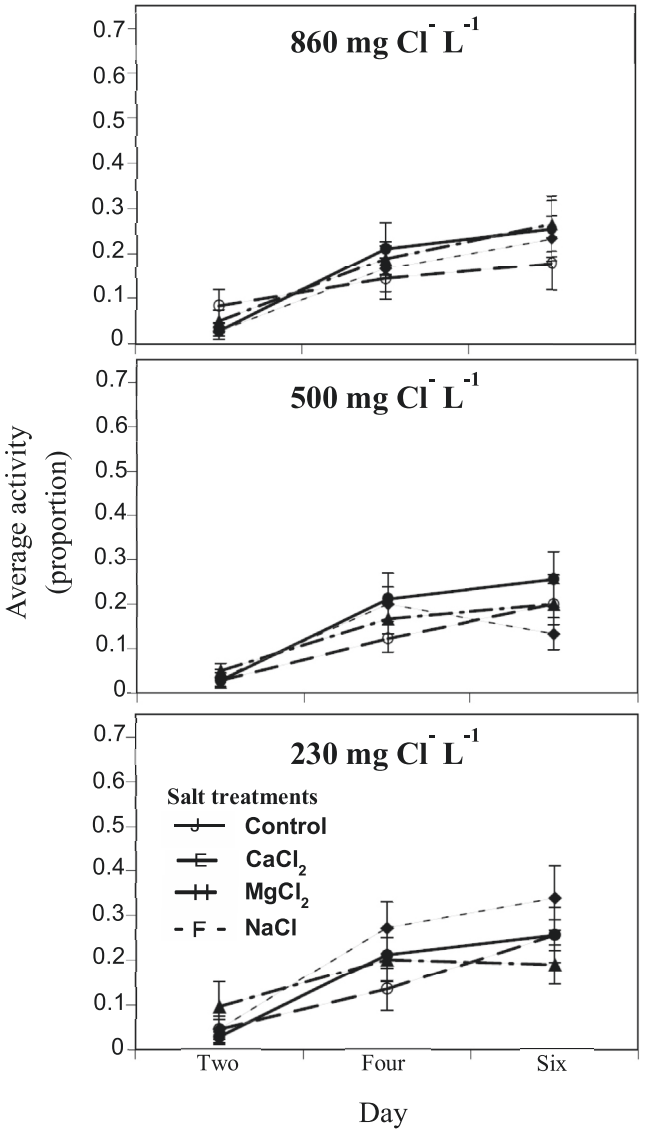

Fig. 3. Average proportion of activity displayed by tadpoles (±SE) across different days exposed to three salt types: $CaCl_2$, $MgCl_2$ and NaCl, across three Cl^- concentrations: 230, 500 and 860 mg Cl^- L^{-1} and a control (25 mg Cl^- L^{-1}).

($F_{1, 110}$, $p < 0.001$). There were no significant interactions with time on tadpole activity ($p > 0.05$ for all).

We found a significant overall effect of our model on tadpole activity for day 7, where activity was measured before and after tadpoles were exposed to echinostome cercariae ($F_{19, 220}$, $p = 0.018$; Fig. 4). We found significant main effect of cercariae exposure on tadpole activity ($p = 0.001$), where tadpoles exposed to cercariae increased their activity by 17% on average relative to their activity prior to exposure. We found no main effect of salt type ($p = 0.066$), or Cl^- concentration ($p = 0.082$) on tadpole activity. There were no significant interactive effects between salt type and Cl^- concentration ($p = 0.686$), between cercariae exposure and Cl^- concentration ($p = 0.606$), between cercariae exposure and salt type ($p = 0.606$) or between salt type, Cl^- concentration and cercariae exposure ($p = 0.749$) on tadpole activity.

4. Discussion

We evaluated the effects of NaCl, $MgCl_2$ and $CaCl_2$, across multiple Cl^- concentrations, on the host-parasite interactions of larval wood frogs and their trematode parasites. We found that exposure to $CaCl_2$ significantly reduced the survival of cercariae compared to the control, while neither NaCl nor $MgCl_2$ had any effects. Additionally, we found that exposure of larval wood frogs to NaCl, but not $MgCl_2$ or $CaCl_2$, increased the intensity of trematode infections relative to larvae not exposed to added salts, but only at a Cl^- concentration of 500 mg L^{-1}. However, increased trematode infections were not a result of deicing salts limiting parasite avoidance behaviors of the tadpoles as seen in other studies (Milotic et al., 2017).

The infectivity and survival of echinostome cercariae declines rapidly following their emergence from snail hosts (Fried and Graczyk, 1997). For example, cercariae of *Echinostoma trivolvis* are the most infectious within the first 8 h after emergence, with infectivity peaking at hours 6–8 (Fried and Graczyk, 1997). Given the limited window of infectivity for echinostome cercariae, contaminant-mediated reductions of cercariae during this window of time (0–8 h post-emergence) could limit their transmission to tadpole hosts (a density-mediated effect on infection) (Rohr et al., 2008). Here, we found that exposure to $CaCl_2$ reduced the survival of echinostome cercariae across all Cl^- concentrations. We found that cercariae exposed to $CaCl_2$ showed a 35% reduction in survival time compared to cercariae exposed to NaCl and $MgCl_2$ and a 45% reduction in survival time compared to the control. $CaCl_2$ exposure decreased the average lifespan of cercariae to ~4 h in our study. Thus, exposure of echinostome cercariae to $CaCl_2$ at environmentally relevant concentration of Cl^- (230–1000 mg Cl^- L^{-1}) has the potential to dramatically reduce parasite transmission to tadpole hosts. However, neither $MgCl_2$ nor NaCl exposure reduced cercarial survival in a similar manner to that of $CaCl_2$. Similarly, Milotic et al. (2020) report no effect of NaCl exposure (160–960 mg L^{-1}) on the survival of echinostome cercariae. Milotic et al. (2020) posited that the lack of effect of NaCl on survival may be due to cercariae needing to withstand similar concentrations of Na^+ and Cl^- found within the tissues of their amphibian hosts. Evaluation of ions in the blood plasma of northern leopard frogs (*Rana pipiens*), which can serve as hosts for echinostomes (Fried et al., 1997) support this line of thought for Na^+, with average concentrations of 2148 mg L^{-1} Na^+, but not for Mg^{2+}, where concentrations levels were much lower at 18 mg L^{-1} Mg^{2+} (Stewart et al., 2004). Thus, this is not a likely reason for the relative tolerance of the echinostome cercariae seen here to $MgCl^2$. Overall, while the evidence for trematode cercariae being tolerant of NaCl is strong across species (Donnelly et al., 1984; Milotic et al., 2020; Shostak, 1993; Venable et al., 2000), ours is the first to indicate a tolerance of cercariae to $MgCl^2$ exposure as well, but only for echinostomes. Further research is thus needed to determine whether the tolerance to $MgCl^2$ seen here is specific to echinostomes, or is generalizable across trematode species.

While our study did not examine the mechanisms underlying the toxicity of $CaCl_2$ to echinostome cercariae that we saw, we can attempt to draw paralells of our findings with those from the literature on schistosomes, which have long been a model parasite due to their infectivity of humans. Indeed, Ca^{2+} cations have been proposed to be integral to the functioning of both cercariae and adult worms of schistomes. For example, schistosome cercariae secrete protease enzymes to penetrate mammalian hosts (Stirewalt and Austin, 1973), which have been shown to be stimulated *in-vivo* by $CaCl_2$ concentrations below ~111 mg L^{-1} (reported concentrations originally in mM: 10 mM $CaCl_2$), and inhibited at concentrations above these (Dresden and Edlin, 1974). Similarly, the antihelminth drug Praziquantelto, commonly used to treat the disease schistosomiasis in humans, induces paralysis of adult schistosomes by increasing cell membrane permeability,

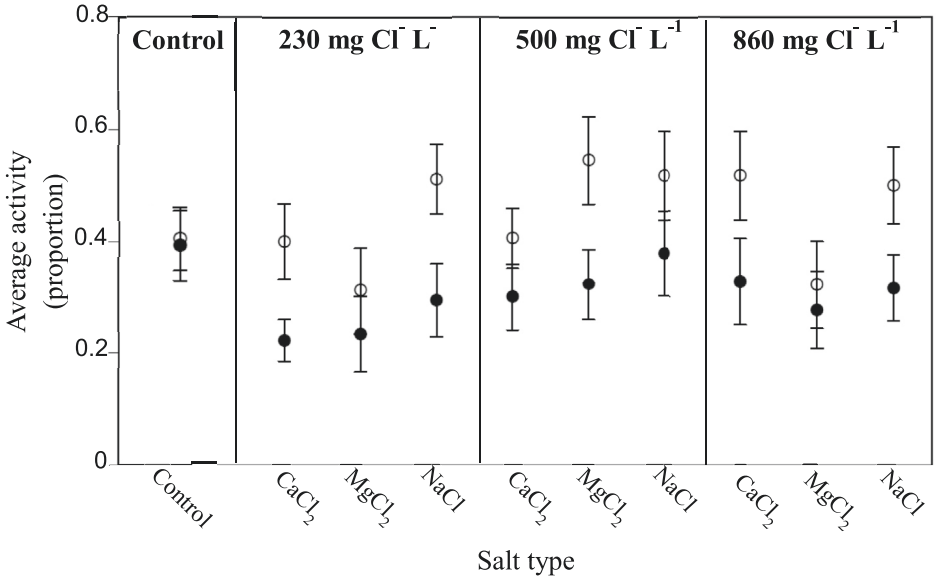

Fig. 4. Average proportion of activity displayed by tadpoles (±SE) before and after being exposed to free-swimming cercariae. Tadpoles and cercariae were exposed to three salt types: $CaCl_2$, $MgCl_2$ and NaCl, across three Cl^- concentrations: 230, 500 and 860 mg Cl^- L^{-1} and a control (25 mg Cl^- L^{-1}).

subsequently allowing for the influx of Ca^{2+} cations which induces contractions of schistosome cell musculature (Cupit and Cunningham, 2015). Similarly, Ca^{2+} binding sites on the glycocolax of the cestode *Hymenolepis diminuta* suggests a role of Ca^{2+} in regulating the integumental structure and function of these worms (Lumsden, 1973). Thus, while we cannot make any definitive conclusions as to the mechanisms underlying echinostome mortality following $CaCl_2$ exposure in our study, the literature suggests a large role for Ca^{2+} cations in the structure and function of cell musculature in trematodes and other platyhelminthes. Thus, it may be that the exposure to Ca^{2+} cations in our study impaired the motor function of the cercariae through such effects on their cell musculature. However, further studies will be needed to evaluate this hypothesis.

Previous toxicology assessments of exposure to deicing salts on wood frog tadpoles suggests that $CaCl_2$ is the most toxic, followed by $MgCl_2$ and NaCl, respectively (Harless et al., 2011). Thus, we predicted that exposure of wood frog larvae to the salts would increase infection intensity the most for those exposed to $CaCl_2$, followed by $MgCl_2$ and NaCl, respectively. Interestingly, we found the opposite. At 500 mg Cl^- L^{-1}, tadpoles exposed to NaCl showed significantly higher levels of infection than tadpoles exposed to the control. Our time-to-death experiment revealed that exposure of cercariae to $CaCl_2$ greatly reduced their survival. Thus, it is likely that the lack of effect of $CaCl_2$ exposure on trematode infection in wood frog hosts seen here is a product of reduced numbers of cercariae able to infect hosts following exposure to $CaCl_2$. Overall, these findings suggest that the underlying mechanisms by which road salts alter host-parasite interactions depend on the lethal and sublethal impacts of the salts on both species.

Exposure to NaCl at a concentration of 500 mg Cl^- L^{-1} increased trematode infection in wood frog hosts. Why exposure to NaCl at 230 or 860 mg Cl^- L^{-1} had no effect is not immediately apparent, but several possibilities exist. For instance, to survive in elevated salinities, freshwater organisms must maintain an osmotic balance between the ions in their cells and those in their environment (Rankin and Davenport, 1981). Physiological shifts to maintain osmotic balance in response to elevated salinity can increase stress hormones such as corticosterone in freshwater organisms (Burraco and Gomez-Mestre, 2016; Degani and Nevo, 1986; Hopkins et al., 2016). Increases in corticosterone can be immunosuppressive, altering interactions between hosts and their parasites (Kaiser et al., 2015; LaFonte and Johnson, 2013). For example, gray tree frog larvae (*Hyla versicolor*) exposed to exogenous corticosterone resulted in an increase in susceptibility to trematode parasites by as much as three times compared to individuals not exposed to corticosterone (LaFonte and Johnson, 2013). Thus, exposure to 500, but not 230 mg Cl^- L^{-1}, may be a sufficiently high enough concentration of Cl^- to increase corticosterone levels, resulting in the increased susceptibility to trematodes seen in our study. Future research needs to examine corticosterone levels of wood frogs across multiple Cl^- concentrations and salt types to evaluate this hypothesis.

The traditional toxicology paradigm is that "the dose makes the poison"; thus, exposure to Cl^- concentrations above 500 mg Cl^- L^{-1} should have further increased trematode infections in wood frog tadpoles. Despite this, exposure of wood frogs to 860 mg Cl^- L^{-1} did not suffer from increased trematode infections relative to the control. Our cercariae time-to-death experiment showed an effect of salt type, but not Cl^- concentration on cercariae survival. Thus, density-mediated effects of increased Cl^- concentration on cercariae (reductions in abundance) are not a likely cause for these findings. However, our experimental design did not test for sublethal effects of deicing salt exposure on echinostome cercariae infectivity, or for the possibility of the Cl^- concentrations used here to act non-monotonically on host susceptibility to infection as has been done for other contaminants (Buss et al., 2019; Rohr et al., 2008). Indeed, experimental designs that expose hosts and parasites to contaminants in isolation of one another can help to tease apart the effects of contaminant exposure on host-parasite outcomes. For instance, Koprivnikar et al. (2007) found that sublethal exposure of *E. trivolvis* cercariae to the pesticide atrazine reduced their ability to successfully infect unexposed wood frog tadpoles. Conversely, the authors found that sublethal exposure of wood frog tadpoles to atrazine increased their susceptibility to unexposed *E. trivolvis* cercariae. When both hosts and parasites were exposed, Koprivnikar et al. (2007) found no difference in infection between atrazine-exposed tadpoles and those not exposed to atrazine. Thus,

future studies that isolate the effects of deicing salts on both host and parasite, similar to those of other investigators, will be needed to further tease apart the effects of deicing salt exposure on infection outcomes within this system.

In the presence of cercariae, anuran larvae display behaviors associated with parasite avoidance, such as increases in activity (Daly and Johnson, 2011; Koprivnikar et al., 2006; Milotic et al., 2017). Exposure to NaCl can reduce the ability of tadpoles to increase their activity when exposed to cercariae (Milotic et al., 2017; Sanzo and Hecnar, 2006). For example, Milotic et al. (2017) found that wood frogs exposed to NaCl concentrations of 600 and 1050 mg L^{-1} NaCl (~364.2 and 637.35 mg Cl^{-} L^{-1}) had reduced activity in the presence of cercariae compared to tadpoles not exposed to added salt. This reduction of activity led to increased infection relative to individuals not exposed to added salt. Thus, for tadpoles exposed to NaCl (500 mg Cl^{-} L^{-1}) in our study, where exposure increased the intensity of trematode infections, we hypothesized that there would be 1) an overall reduction in activity of tadpoles exposed to NaCl relative to the control and/or 2) a reduced ability of tadpoles to mount a behavioral response to cercarial exposure (i.e. no change in tadpole activity between pre − and − post − cercariae exposure) relative to the control. However, we found no effect of salt exposure on tadpole activity relative to the control. Further, unlike Milotic et al. (2017) salt exposure did not impact the ability of tadpoles to mount a behavioral response against cercariae. Indeed, tadpoles in our study increased their activity in the presense of cercariae regardless of salt exposure. It is unlikely that the reason for these differential findings is due to differences in Cl^{-} exposure as the concentrations used here are greater than those of Milotic et al. (2017). However, Milotic et al. (2017) exposed wood frogs for a greater length of time than our study (7 versus 12 d). This suggests that the length of exposure to salts may play a role in mediating the influence of salt exposure on tadpole activity. We did not find a salt-by-day interaction, indicating that exposure to the salts did not influence activity differentially across the 7 d of our study. Thus, we may not have exposed wood frog tadpoles long enough to see an effect of salt exposure on activity. However, we also found an increase in overall tadpole activity across time. This was likely due to tadpoles being on a fixed food ration. Thus, it may be possible that the increased activity across our study may have masked the effects of salt exposure on tadpole activity. Lastly, our scan sampling methodology differs from those of others who have investigated the presence of cercariae on tadpole activity using video recording devices (Marino, 2016; Milotic et al., 2017; Sears et al., 2015). For instance, Sears et al. (2015) recorded tadpoles for 10 min after exposing them to cercariae and measured several behavioral responses hypothesized to be associated with parasite avoidance, such as swimming speed and angle of movement, as well as “evasive” maneuvers such as explosive swimming with erratic changes in direction. Thus, it is possible that our measurement of activity alone, and shorter period of observation than those of others may have been inadequate to capture the full range of possible effects of the road salts on tadpole behavior in the presence of the cercariae. Ultimately, further research which evaluates tadpole behavior across greater periods of time in response to salt exposure and with more precise measurements, such as those mentioned above, will be needed to better understand the differential outcomes of exposure on tadpole activity between our study and Milotic et al. (2017).

Future considerations: Our findings and those of others (Buss and Hua, 2018; Milotic et al., 2017) show that exposure of amphibian larvae to environmentally relevant concentrations of NaCl across a range of exposure periods (1–12 d of exposure) increases the intensity of trematode infections in NaCl-exposed hosts. While the individual effects of exposure to salts and trematode infections on amphibian larvae are fairly well understood (Harless et al., 2011; Orlofske et al., 2017; Sanzo and Hecnar, 2006), how the two stressors interact to influence key metrics of amphibian fitness, such as growth, time-to-metamorphosis and survival are not. Thus, future research is needed to understand how exposure to deicing salts and trematode infection interact to influence amphibian populations and communities. By investigating how the most common road deicing salts (NaCl, $MgCl_2$ and $CaCl_2$) interact with parasitic infections, we will be better able to manage and conserve the health of amphibian populations near roadways.

Further, given the strong positive relationship between planorbid snail density − which serve as the first intermediate host of echinostomes - and trematode infections in amphibians (Skelly et al., 2006) an investigation of how NaCl, $CaCl_2$ and $MgCl_2$ each influence survival and reproductive output of planorbid snails across various Cl^{-} concentrations is warranted. For example, were road salt exposure to decrease survivorship and/or reduce fecundity of snail populations, this could greatly reduce trematode transmission from snails to larval amphibians. With regard to NaCl, exposure of trematode-infected planorbid snails to a concentration of 2500 mg L^{-1} (but not 250 or 1250 mg L^{-1}) greatly reduced their survival in aquaculture ponds, effectively removing the cercarial life stage of the trematode *Bolbophorus confusus* from the ponds (Venable et al., 2000). However, whether these results are generalizable to $CaCl_2$ or $MgCl_2$ is unknown. Additionally, cercarial output from marine snails has been shown to differ under varying levels of salinity (Koprivnikar and Poulin, 2009; Lei and Poulin, 2011), suggesting the possibility for similar effects of salinity on the emergence of freshwater cercariae from snail hosts. Thus, an evaluation of cercarial shedding from snails exposed to road salts across multiple Cl^{-} concentrations will be necessary, as altered cercarial output could affect transmission to amphibian hosts.

5. Conclusions

To sum, we found that exposure to $CaCl_2$ greatly reduced trematode survival across all Cl^{-} concentrations (230, 500, 860 and 1000 mg Cl^{-} L^{-1}) while neither NaCl nor $MgCl_2$ had any impact. Exposure of both parasites and hosts to the salts resulted in increased infection for hosts exposed to NaCl, but not $MgCl_2$ or $CaCl_2$. However, salt exposure only increased infection at 500 mg Cl^{-} L^{-1}, having no impact on infection at 230 or 860 mg Cl^{-1} L^{-1}. Increased infection at 500 mg Cl^{-} L^{-1} was not due to NaCl reducing the activity of tadpoles in the presence of parasites, suggesting a role for salt-mediated changes to immunocompetence (Mangahas et al., 2019). Overall, these findings highlight the need to consider impacts of contaminant exposure on both host and parasite when making predictions of exposure on host-parasite outcomes. By accounting for toxicity of contaminants to both host and parasite, investigators will be better equipped to predict the net outcomes of contaminant exposure on host-parasite interactions.

CRediT authorship contribution statement

Nicholas Buss: Conceptualization, Methodology, Investigation, Validation, Formal analysis, Data curation, Writing - original draft, Writing - review & editing, Visualization. **Kiersten N. Nelson:** Conceptualization, Methodology, Investigation. **Jessica Hua:** Conceptualization, Methodology, Writing - original draft, Writing - review & editing, Supervision, Project administration, Funding acquisition. **Rick A. Relyea:** Conceptualization, Methodology, Writing - original draft, Writing - review & editing, Supervision, Project administration, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envpol.2020.115244.

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