RESEARCH ARTICLE

Salinity increases growth and pathogenicity of water mold to cause mortality and early hatching in *Rana sylvatica* embryos

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One sentence summary: Water mold, a common freshwater pathogen, infects and kills frog embryos and causes increased premature hatching when water also contains road salt, a common freshwater pollutant.

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ABSTRACT

Amphibian embryos often suffer increased mortality and altered hatching when exposed to road deicing salt runoff or pathogens such as water molds. However, the combined effects of such contaminants on embryos remain understudied. To test how pond salinization interacts with water mold (*Saprolegnia* sp.) to influence hatching timing and survival, we first measured pond water conductivity and temperature and quantified the prevalence and abundance of water mold in four ponds in an ecological preserve. Second, we experimentally placed wood frog (*Rana sylvatica*) embryos in the presence or absence of water mold, crossed with environmentally realistic salt concentrations (100, 300 or 600 μS). Lastly, we quantified growth and colonization of water mold in this range of salinities. Our results demonstrate that salt had synergistic effects with water mold exposure that affected hatching time, though water mold had less of an effect at higher salinities. Water mold significantly reduced egg survival whereas salt did not. Higher salinities also increased water mold growth and colonization on new substrates. These results indicate that road salt runoff may enhance colonization of amphibian eggs by water molds increasing mortality and premature hatching of surviving embryos, which may in turn have detrimental effects on amphibian communities.

Keywords: *Saprolegnia*; pollution; road salt; Anura; freshwater ecology; induced hatching

INTRODUCTION

Anthropogenic changes to the environment, such as excessive land use (Brooks *et al.* 2002; Foley *et al.* 2005; Chaudhary, Pourfaraj and Mooers 2018), fossil fuel usage contributing to climate change (IPCC 2014; Pimm *et al.* 2014) and pollution (Gilbert 2017), are altogether decreasing global biodiversity. In aquatic ecosystems, chemical pollutants in particular can prove detrimental to the health, development and survival of the organisms that inhabit these environments (Foley *et al.* 2005; Gilbert 2017). For example, wild Pacific salmonids no longer use 40% of

their historical breeding ranges, which is partially attributed to chemical accumulation in sediments that weakened the salmons' immune systems, leaving them more susceptible to disease-causing pathogens (Arkoosh *et al.* 1998). In addition, chemical contaminants can cause abnormal development. Snapping turtle (*Chelydra serpentina serpentina*) eggs in the Great Lakes Basin exposed to polychlorinated biphenyls (PCBs) have decreased hatching success and increased deformities (Bishop *et al.* 1998). Though the manufacturing of PCBs was banned in 1978, contaminants in snapping turtle eggs are still found in Lake Ontario, the St Lawrence River, and their connecting

channels, with increasing contamination near urban/industrial centers (de Solla *et al.* 2007).

Aquatic chemical disturbances especially affect amphibians due to their highly permeable skin, which allows gas, water and electrolyte exchange with the environment (Brühl *et al.* 2013). Because amphibians migrate between both aquatic and terrestrial habitats, their risk of toxicant exposure is higher than that of organisms adapted to inhabit strictly water or land. This risk, coupled with unavoidable dermal uptake of chemicals, makes amphibians vulnerable to changes in their environments (Brühl *et al.* 2013).

In addition to the detrimental effects of chemical pollutants, amphibian population declines and localized extinction events have been linked to aquatic pathogens such as water molds. Water molds are parasitic oomycetes (but are unrelated to true fungi) and are common in freshwater systems where they infect amphibian eggs both through direct contact and growth of hyphae as well as through colonization from free-floating spores (Diéguez-Uribeondo, Cerenius and Söderhäll 1994; Green 1999; Fernández-Benéitez *et al.* 2008). Numerous genera of water molds contain species known to be pathogenic to amphibians: *Saprolegnia*, *Leptolegnia*, *Achlya*, *Aphanomyces* and *Pythium* (Ault *et al.* 2012; Ruthig and Provost-Javier 2012). Infections by common species of *Saprolegnia* such as *S. ferax*, *S. parasitica* and *S. diclina* across North America, Europe and Asia are particularly detrimental to the survival of amphibians at early life stages (Lefcort *et al.* 1997; Robinson, Griffiths and Jeffries 2003; Touchon, Gomez-Mestre and Warkentin 2006; Fernández-Benéitez *et al.* 2008; Romansic *et al.* 2009; Ruthig 2009; Groffen *et al.* 2019) and have been implicated as possibly contributing to declining numbers of some species (Blaustein *et al.* 1994; Green 1999).

The embryos of many amphibians are capable of responding to environmental risks and stressors by hatching prematurely (Warkentin 2011), and in particular, several amphibian species are known to hatch early in response to water mold infection (Gomez-Mestre, Touchon and Warkentin 2006; Touchon, Gomez-Mestre and Warkentin 2006). Although early hatching increases short-term survival, these responses can be unfavorable over the longer term, as underdeveloped hatchlings are potentially more susceptible to predators and disease (Gomez-Mestre, Touchon and Warkentin 2006; Warkentin 2011; Touchon *et al.* 2013). Additional environmental stressors, such as UV exposure, can increase susceptibility of egg masses to water mold invasion, increasing egg mortality (Kiesecker and Blaustein 1995). Relatively few studies have investigated synergistic effects of environmental changes and water mold infection on amphibians (e.g. Karraker and Ruthig 2009). However, the few that have found that lower water temperature and the presence of water mold have shown higher egg mortality rates in amphibians like American bullfrogs (*Rana catesbeiana*) and common frogs (*Rana temporaria*) (Beattie, Aston and Milner 1991; Ruthig 2013).

Beyond natural variation in environmental stressors, chemicals transported from roadways often impact amphibians, as vehicles emit toxic pollutants that may ultimately collect in roadside ponds (Karraker and Ruthig 2009). In recent years, the environmental impact of road deicing salt in the United States has gained attention, as roughly 14 million tons of road salt are applied to US roads annually (Sanzo and Hecnar 2006) and salt runoff can travel at least 170 m into roadside wetlands (Karraker and Ruthig 2009). Consequently, increases in road construction and traffic volume are thought to be increasing the salinity of aquatic systems, posing harmful threats to inhabiting organisms. Wood frog (*Rana sylvatica*) embryos and tadpoles inhabiting roadside vernal pools that were contaminated with

deicing salt runoff showed increased mortality at high salinities (Karraker, Gibbs and Vonesh 2008). Additionally, *R. sylvatica* tadpoles exposed to increasing salinity showed decreased survivorship, weight and activity, as well as increased developmental abnormalities (Sanzo and Hecnar 2006). Similar negative effects have been observed in brown tree frog (*Litoria ewingii*) tadpoles (Chinathamby *et al.* 2006), spotted salamander (*Ambystoma maculatum*) embryos (Turtle 2000) and rough-skinned newt (*Taricha granulosa*) embryos (Hopkins, French and Brodie 2013a). Although it is clear that increased salinity reduces embryonic and larval survival in many amphibians, it is unclear how salinity influences timing of hatching. Furthermore, the potential effect of roadside salt contamination and freshwater pathogens also remains understudied.

This study aims to illuminate how increased salinization of wetlands due to runoff from road salts interacts with pathogenic water mold to influence mortality and hatching time of frog eggs. We experimentally tested the independent and interacting effects of increased salinity and exposure to water mold (Saprolegniaceae) on the timing of hatching and survival to hatching. We also quantified the ability of water mold to grow and spread in increasingly salinized water. We hypothesized that increased salinity would act synergistically with water mold, resulting in early hatching and increased egg mortality, and that this would principally be driven by the increased ability of water mold to grow in more saline water. This experiment was informed and supplemented by two years of field survey data collected from four ponds in the Hudson Valley of New York.

MATERIALS AND METHODS

Location and prevalence of water mold in the field

This study was conducted on Vassar College's Farm and Ecological Preserve (VFEP) in Poughkeepsie, NY, USA. Field surveys were performed weekly for eight consecutive weeks between March and May 2017 and for five consecutive weeks in April and May 2019. In both years, field surveys began once ponds first began to thaw and were inclusive of the breeding season of *R. sylvatica*. We selected four ponds within the VFEP for water quality monitoring and water mold quantification (Fig. 1). Two ponds were near the edge of the preserve and were relatively closer to paved roads, whereas two were located more centrally in the preserve. These locations were intentionally chosen to compare ponds with varying proximity to roadways and subsequent potential for road salt runoff. Although the four ponds differ in surface area, they are generally consistent in terms of depth and are surrounded by oak and maple forest with substantial emergent vegetation. The exception in terms of depth is Turtle Pond, which is substantially deeper than the other three. Each pond has a muddy substrate and substantial emergent grass vegetation. The two ponds near the edge of the preserve (Turtle and Zach's Way) are permanent whereas the two ponds more central to the preserve (Big Ditch and Little Ditch) are semi-permanent, generally holding water from spring through most of the summer and fall, depending on rainfall. In addition to wood frogs, all ponds are known to be used by spring peepers (*Pseudacris crucifer*) for breeding, as well as other amphibian species later in the year. To measure water conductivity and temperature levels in each pond, we used a YSI Pro Plus meter in 2017 and a Vernier LabQuest salinity and conductivity meter in 2019. Salinity refers generally to the presence of salts in water, whereas conductivity refers to the actual ability of electricity to move

Figure 1. Map of ponds sampled at the VFEP. Conductivity ranged from highest to lowest in Zach's Way, Turtle, Little Ditch and Big Ditch, respectively. Turtle pond is located directly downhill (∼200 m) from residential housing, and Zach's Way lies fewer than 20 m from a major roadway. Little Ditch and Big Ditch are more secluded and receive little road runoff. Image from Google Earth. The road in the center of the VFEP is a dirt road with restricted traffic. In winter, the dirt road in the VFEP is only maintained through plowing, whereas the paved roads surrounding the VFEP are plowed and have road salt added.

through water because of salt ions. Measurements were consistently taken approximately two feet from the shoreline of each pond in the top 6–8 inches of the water column (water depth in this part of the ponds ranged from 12 to 18 inches), during the mid-afternoon, and in similar locations each week. These locations were chosen based on accessibility so that we could accurately replicate the measurements each week.

Water mold identification and culturing

Water mold was collected from each pond by submerging paper teabags containing sterilized (boiled) hemp seeds and a small rock, which ensured complete submersion (Robinson, Griffiths and Jeffries 2003). To quantify water mold growth across ponds, we prepared bags with 10 sterile seeds each and submerged them for four (2017) or seven (2019) days. Bags were placed in the same location where conductivity and salinity levels were measured each week. Once submerged for the appropriate time interval, the bags were brought to the lab, and we randomly selected one seed from each bag to plate on a 30-mm Petri dish filled with autoclaved corn meal growth agar (Sigma-Aldrich, St. Louis, MO, USA). Using a stereo microscope, we outlined terminal hyphal growth on the Petri dish after 24 h in a room temperature laboratory (generally 21°C). We photographed each Petri dish with a ruler and used ImageJ (Rasband 2012) to quantify the area of growth per seed. Petri dishes were always assigned random numbers visible in each photograph to ensure that measurements of hyphae growth were taken blindly with respect to the pond of origin.

To generate infected seeds for laboratory experiments, excess seeds from Turtle pond (Fig. 1) were mixed with additional sterilized seeds and plated on 60-mm Petri dishes of autoclaved corn meal growth agar to encourage hyphal growth. Seeds were incubated at room temperature for 72–96 h prior to the start of exposures.

Effects of salt and water mold on embryo hatching and survival

On 25 March 2019, 10 *R. sylvatica* egg clutches were collected within 24 h of oviposition from the Big Ditch pond on the VFEP (Fig. 1), and eggs were incubated at 4°C overnight to slow their development. Six groups of 10 eggs each were removed from each egg clutch using sterilized forceps. Eggs that were not used for the experiment were returned to the pond unharmed. The population of *R. sylvatica* on the VFEP lays upward of 300 egg clutches each spring (Touchon unpublished data), with egg clutches containing ∼700 eggs each (Martof 1970), so this experiment had little overall effect on our population. Experiments began before the eggs reached Gosner stage 10 (Gosner 1960).

This experiment aimed to quantify the combined effects of salt and water mold on *R. sylvatica* egg survival and hatching. To prepare each salt water concentration, we mixed 16 L of reverse osmosis (RO) water with 0.80 g of R/O Right (Kent Marine, Franklin, WI, USA) to obtain a stock of artificial pond water (100 μS), hereafter referred to as the low salt treatment. To create the medium and high salt treatments (300 and 600 μS), we added 0.09 or 0.25 g/L of halite road salt (NaCl) to the stock artificial pond water, respectively. These values represent the range of conductivities measured across all ponds at the VFEP in 2017 and 2019 (see the 'Results' section). Experiments were conducted in 473-mL transparent polypropylene plastic cups with sealable lids. We filled each cup with 200 mL of appropriate water. Salinity treatments were crossed with the presence or absence of a

sterilized hemp seed infected with water mold, resulting in six different treatment combinations. Each cluster of 10 eggs within each cup was a separate replicate, resulting in a total sample size of 60 experimental units. When setting up the experiment we took care to ensure that each seed, and therefore the hyphae on it, made contact with the eggs. A sterilized control seed was placed with eggs in the no water mold control treatment. Cups were sealed with a lid to ensure minimal evaporation, and cups were placed into an environmental chamber in a fully randomized design on two shelves (Conviron, Pembina, ND, USA). Water was not refreshed during the experiment. The environmental chamber maintained a 12:12 h light:dark schedule at 14°C and 12°C, respectively. These temperatures reflect data measured at the collection site just after *R. sylvatica* oviposition events. All experiments were checked daily at 1200 h for hatched, unhatched, and dead eggs. Any mortality noted in the first 24 h of the experiment was attributed to handling effects. Eggs were considered hatched when they had completely exited the egg capsule. We did not remove dead eggs or hatchlings from cups during the course of the experiment and dead eggs did not appear to have detrimental effects on water quality in any cups. Upon completion of the study, hatchlings not exposed to water mold were released at the site of original collection, and those that were exposed to water mold were euthanized in 1.0 g/L neutral buffered MS-222. All water mold-associated waste was autoclaved before disposal.

Water mold growth and colonization in varying salinities

In the fall of 2019, water mold was collected from ponds at the VFEP as described above. Three salt water concentrations (100, 300 and 600 μS) were prepared as above, and 50 mL of each solution was distributed among 30 glass jars, each 60 mm in diameter ($N = 10$ per salt concentration). One hemp seed covered in visible water mold hyphae was randomly chosen and placed into the center bottom of each jar. After sitting for 24 h, two sterilized seeds were placed opposite of each other at the edges of the bottom of each jar. Jars were sealed with parafilm to prevent evaporation and were incubated at room temperature (21°C) for 48 h, at which point the three seeds were plated on 30-mm Petri dishes containing autoclaved corn meal growth agar. After the 24-h period, the terminal hyphal growth around each seed was traced under a dissecting scope. Photographs were then taken of all 90 traced plates and the hyphae growth outlines were measured in ImageJ. Petri dishes were assigned random numbers visible in each photograph to ensure that measurements of hyphae growth were taken blindly with respect to the salt water treatment and if seeds were originally or secondarily infected.

Statistical analysis

All statistical analyses were conducted in R 3.6.1 (R Development Core Team 2019). Field measurements of conductivity and temperature in different ponds collected in 2017 and 2019 were analyzed with linear mixed-effects models (LMMs), including site and week as fixed effects (predictors) and year as a random effect to control for possible interannual variation (Bates *et al.* 2015). In all mixed-effects models, here and those described below, significance of predictors was estimated using likelihood ratio tests of nested models. Model fit was assessed using quantile–quantile plots of residuals.

Water mold area was log-transformed in all analyses of water mold growth to increase linearity and model fit. Water mold
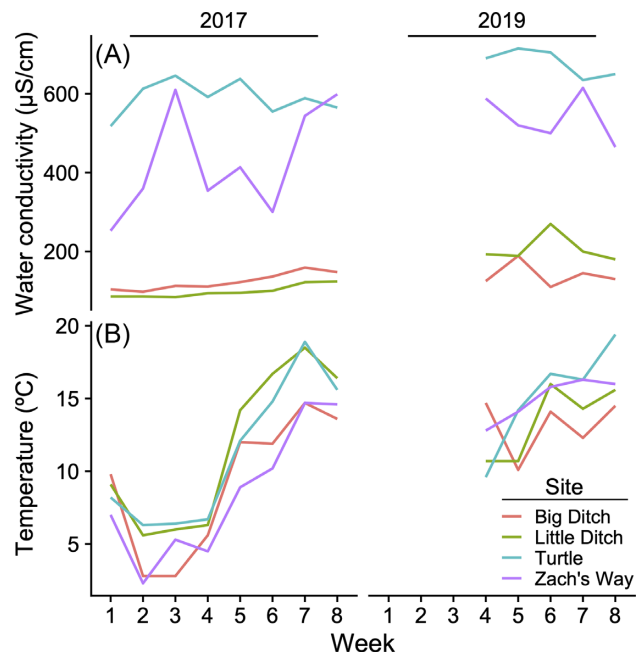

Figure 2. Conductivity and temperature changes over eight weeks. **(A)** Temperature steadily increased over time in both 2017 and 2019, whereas **(B)** conductivity remained relatively consistent over time (both within and across years) but varied considerably across sites. Note that data were not collected during the first three weeks of 2019 due to a longer winter wherein ponds were still frozen and frogs were not yet breeding.

growth across ponds sampled at the VFEP was analyzed with LMMs, including week and week nested within year as random effects. Our analysis of water mold growth from the field contained three predictors (site, temperature and conductivity) and all their interactions.

Effects of water mold and salinity treatments on average hatching time in the laboratory experiment were analyzed using LMMs, including shelf and family as crossed (i.e. independent) random effects. To understand the nature of a significant interaction between water mold infection and salinity treatment on hatching time (see the 'Results' section), we conducted Tukey post-hoc analyses using the emmeans package (Lenth 2020). Survival in the laboratory experiment was analyzed with a generalized linear mixed-effect model (GLMM) with a binomial error distribution, also including shelf and family as crossed random effects.

LMMs were used to measure the effect of salinity on water mold growth and of water mold colonization on new seeds. Each jar was an independent replicate, but random effects were used to control for the non-independence of the three seeds (one infected and two originally uninfected) in each jar. Tukey post-hoc analyses to compare among salinity levels were conducted in the emmeans package (Lenth 2020).

RESULTS

Quantifying conductivity, temperature and water mold in the field

Field survey data revealed that conductivity differed significantly between the four ponds (Fig. 2; $\chi^2 = 130.5$, $P < 0.0001$), with the highest conductivity levels measured in the Turtle and Zach's Way ponds, both of which lie relatively close to major roadways and residential areas (Fig. 1). Conductivity levels were

Table 1. Results of linear mixed-effect models analyzing the effects of site, water temperature and water conductivity on water mold growth. Water conductivity and temperature were measured weekly during the spring months of 2017 and 2019. Water mold was collected from each pond via sterilized hemp seed baits and was grown in the lab to quantify the relative amount present. Random effects included in the model were the week the sample was taken nested within the year of the sample. P-values were obtained via likelihood ratio tests of nested models.

Predictor	χ^2	P-value
Site	26.99	<0.0001
Conductivity	5.27	0.02
Temperature	0.02	0.90
Site × temperature	11.76	0.008
Site × conductivity	15.77	0.001
Temperature × conductivity	0.05	0.82
Site × temperature × conductivity	5.78	0.12

between ∼100 and 700 μS, with occasional values as high as 715 μS. Additionally, there was a marginally significant effect of week on conductivity ($\chi^2 = 3.3$, $P = 0.07$) but no interaction effect between site and week (Fig. 2A; $\chi^2 = 5.2$, $P = 0.16$). Pond temperature increased over time (Fig. 2B; $\chi^2 = 50.9$, $P < 0.0001$) and did not differ significantly between sites ($\chi^2 = 5.7$, $P = 0.13$).

Water mold was found in all ponds surveyed, and the amount of growth cultured in the lab differed significantly between sites as well as with increasing conductivity (Table 1). More importantly, water mold growth significantly increased with both increasing conductivity and temperature, even when accounting for differences in sites (Fig. 3; Table 1). There was no significant three-way interaction between site, conductivity and temperature.

Effects of salt and water mold on embryo hatching and survival

As expected, water mold caused *R. sylvatica* eggs to hatch early across all salt treatments (Fig. 4A; $\chi^2 = 27.11$, $P < 0.0001$). Furthermore, increasing salinity also caused eggs to hatch early with the earliest hatching occurring in the highest salt concentration ($\chi^2 = 32.98$, $P < 0.0001$). There was also a significant interaction effect between salt and water mold, such that the presence of water mold had less of an effect on hatching time as salt levels increased ($\chi^2 = 5.81$, $P = 0.05$). Tukey post-hoc analyses revealed that the hatching age of control embryos versus those exposed to water mold differed significantly in the 100 and 300 μS concentrations (both $P \leq 0.0001$), but were only marginally different in the 600 μS concentration ($P = 0.08$).

Water mold infection increased egg mortality across all salinity treatments (Fig. 4B; $\chi^2 = 14.04$, $P = 0.0002$), while salt concentration had no lethal effects ($\chi^2 = 1.93$, $P = 0.38$). There was no interaction between salinity and water mold on embryo mortality ($\chi^2 = 0.96$, $P = 0.62$). Fifteen tadpoles died after hatching, all of which had hatched in the presence of water mold. Post-hatching mortality did not appear to be affected by salt treatment.

Effect of conductivity on water mold growth and colonization

After incubating infected and non-infected seeds in differing salinities, we found significant effects of both water conductivity and seed type (originally infected vs non-infected) on the

area of water mold hyphal growth after 48 h (Fig. 5; seed: $\chi^2 = 18.62$, $P = 0.00002$; salinity: $\chi^2 = 11.80$, $P = 0.002$). Although water mold growth was higher on originally infected seeds than non-infected seeds, for both seed types water mold growth was greatest in the highest salt concentration and growth did not differ between the two lower concentrations (Tukey post-hoc analyses: infected seeds, 600 vs 100: $P = 0.046$, 600 vs 300: $P = 0.044$, 100 vs 300: $P = 0.98$, clean seeds, 600 vs 100: $P = 0.05$, 600 vs 300: $P = 0.005$, 100 vs 300: $P = 0.68$).

DISCUSSION

Due to their highly permeable skin, amphibians are incredibly vulnerable to changes in their environment. Increased salinity in roadside ponds from deicing salt runoff can have both lethal and sublethal effects on frog embryos, and pathogens such as water mold within these ponds can infect amphibian eggs, causing mortality and early hatching. To determine the prevalence of water mold and salinity in the environment, we performed a field study followed by two laboratory studies, where we first tested the effect of increased salinity and water mold on *R. sylvatica* egg mortality and hatching timing, and secondly examined water mold growth and colonization ability under increasing salinity. In short, we found that water mold was widespread in the environment, that increased pond salinization was associated with an increase in water mold growth and colonization of new substrates, and that water mold and salt had strong effects on *R. sylvatica* embryos, reducing both hatching time and embryo survival.

To gain a better understanding of the salt concentrations and water mold prevalence in ponds in the field, we measured water conductivity and temperature and cultured water mold from four ponds on an ecological preserve across the springs of 2017 and 2019. Conductivity levels differed between sites, which appeared largely driven by each pond's varying proximities to roadways (Fig. 1). Turtle and Zach's Way ponds had the highest conductivities—consistently 3–10 times greater than at the Big Ditch and Little Ditch ponds—and lie ∼200 m downhill from a densely populated residential area and fewer than 20 m from a busy paved road, respectively. This proximity to roadways likely results in increased levels of salt leaching into the ponds and surrounding soil throughout the winter and early spring. The Big Ditch and Little Ditch ponds are located closer to the center of the VFEP (∼500 and 350 m from paved roads, respectively) and are likely more protected from road and residential salt runoff. These two ponds also do not hold water year round, but there is no obvious reason why pond permanence would influence water conductivity.

Although conductivity differed across these four sites, water mold was present in all ponds and showed higher levels of growth with increasing pond conductivity and temperature (Fig. 3). The positive association between growth and conductivity was nearly identical in ponds with significantly different levels of salinization, such as Little Ditch and Turtle ponds, potentially indicating that strains of water mold are locally adapted to each pond. We further corroborated in the lab that increased salinity enhanced both water mold growth and transmission to new substrates (Fig. 5). In addition, factors such as UV-B radiation (Kiesecker and Blaustein 1995) can have synergistic effects with water mold, but, to the best of our knowledge, this is the first documentation of increased conductivity positively affecting water mold growth. In fact, Ali (2005) found that growth of the water mold *Saprolegnia parasitica* decreased with
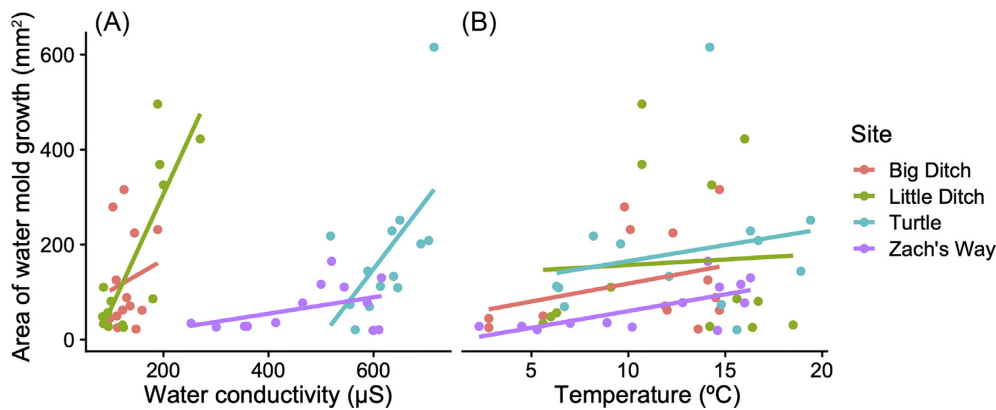
Figure 3. Water mold growth, conductivity and temperature across sites. There was a positive relationship between water mold growth and **(A)** conductivity (μS) and **(B)** temperature ($^\circ$C). Lines represent the means of data collected at the VFEP between March and May in 2017 and 2019.
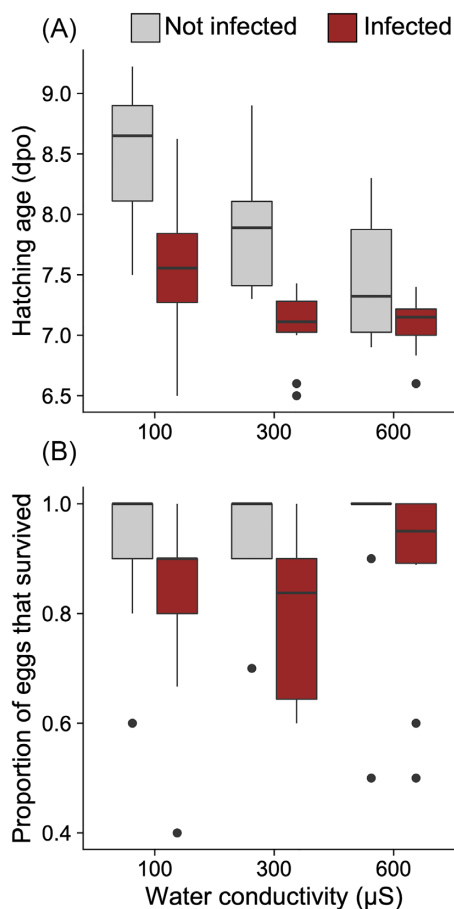

Figure 4. Effects of water mold and salt on embryo hatching and survival. **(A)** Eggs hatched significantly earlier when infected with water mold and in increased salinity. There was a marginally significant interaction effect between salt and water mold infection, such that water mold had less of an effect in high salinity conditions. **(B)** Egg survival was significantly lower in the presence of water mold infection, while no significant effect of salt on survival was detected. The interaction effect between water mold and salt treatments was not significant. Box-and-whisker plots show the median (thick horizontal line), interquartile range (top and bottom of each colored box) and either the most extreme values (ends of the whiskers) or 1.5 times the interquartile range and outliers (ends of the whiskers followed by points).

increasing NaCl concentrations. However, sporangial formation improved when combined with ascorbic acid (Ali 2005), suggesting that perhaps water mold can interact with compounds in the water that promote hyphal growth. Future research is required to understand how different aspects of pond water chemistry influence the prevalence and growth of water mold. It is also worth noting that other studies (e.g. Sanzo and Hecnar 2006; Karraker, Gibbs and Vonesh 2008) have recorded water conductivity as high as 3000 μS—over four times greater than our highest measured level—and it is unknown how such substantially higher levels of salt may impact water mold growth and the effect of water mold on amphibian embryos.

To determine the effect of salinity and water mold on *R. sylvatica*, embryos were placed in either the presence or absence of water mold and crossed with one of three salt concentrations. Both salinity and the presence of water mold caused eggs to hatch early, and the effect of water mold on hatching age decreased as salinity increased. Importantly, while salt did not affect embryo survival itself, water mold exposure (and subsequent infection of some eggs) increased egg mortality, and water mold and salt did not interact to affect survival (Fig. 4). Karraker and Ruthig (2009) also found no interactive effect of salt and water mold on embryo survival, but, unlike the present study, they also found no effect of just water mold on embryo survival. This difference likely stems from the two study's differences in methodology, as Karraker and Ruthig (2009) utilized *A. maculatum* and *R. clamitans* in their laboratory experiments and their eggs were incubated at a relatively higher temperature (20–23°C) compared with the incubation temperature of this study (12–14°C). It is possible that this warmer temperature allowed eggs to develop and hatch before becoming lethally infected by water mold.

Although we found no effect of salt on survival, numerous studies have shown effects of salinity on amphibian survival and development (Karraker and Ruthig 2009). For example, increasingly saline water dramatically reduces survival and increases deformities in rough-skinned newts (Hopkins, French and Brodie 2013a). Research into the effects of salinity on anuran hatching time is limited, but it is noteworthy that embryos of *T. granulosa* also hatch early under elevated salinity (Hopkins, French and Brodie 2013b). Our study documents the first account, to our knowledge, of early hatching by *R. sylvatica* eggs in response to increased salinity.

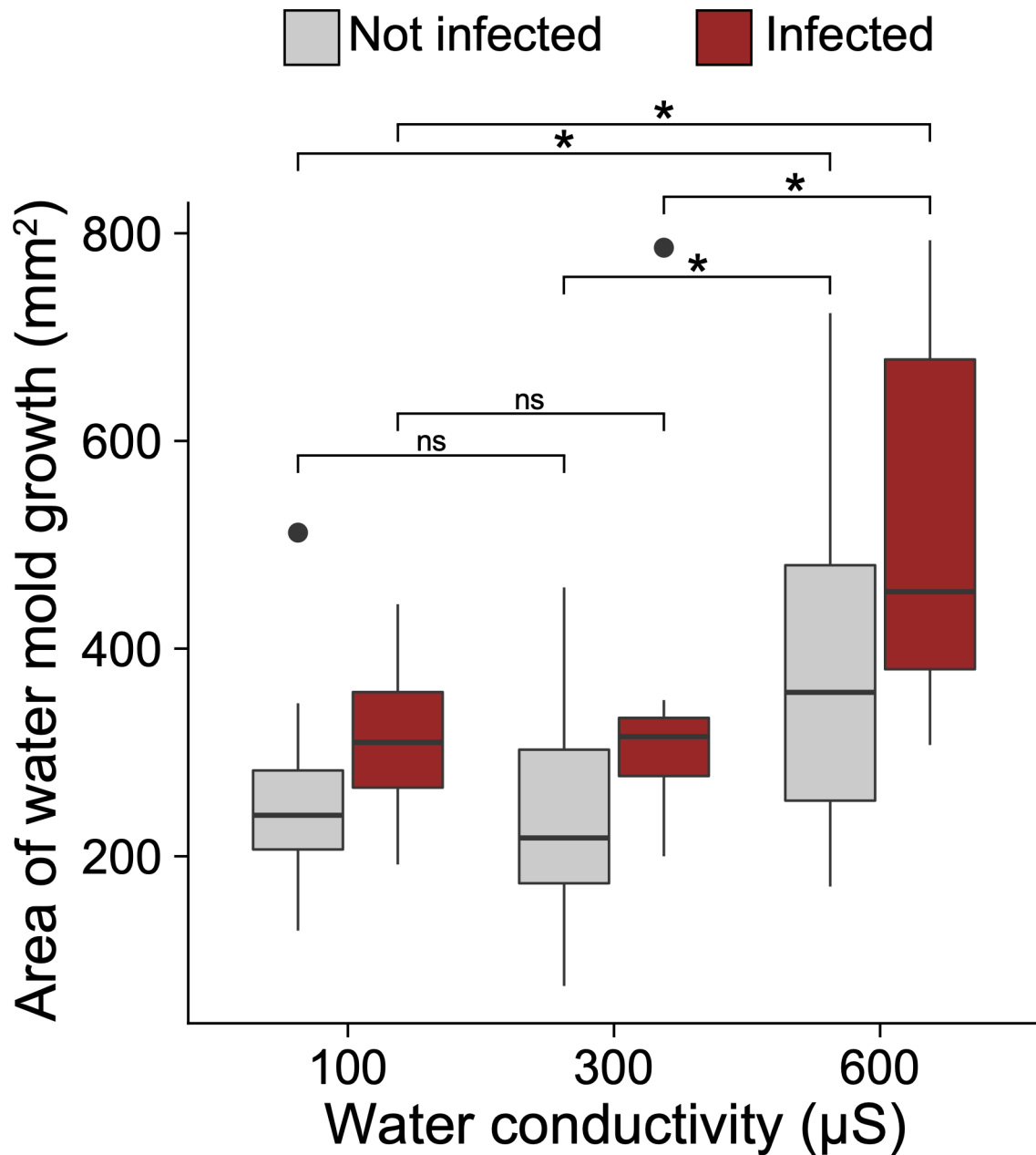The adverse effects of water mold on embryos are more well documented (Green 1999; Gomez-Mestre, Touchon and

Figure 5. Water mold growth and colonization on new substrates under increasing water conductivity. Hyphae growth was significantly greater in the highest salt concentration on both infected and not infected seeds as compared with both of the lower concentrations, whereas growth in the two lower concentrations did not differ. Lines indicate the results of post-hoc comparisons within each seed type. '*' indicates a significant difference ($P \leq 0.05$), whereas 'ns' indicates no significant difference ($P > 0.05$). These patterns were the same for both originally and secondarily infected seeds. Box-and-whisker plots show the median (thick horizontal line), interquartile range (top and bottom of each colored box) and either the most extreme values (ends of the whiskers) or 1.5 times the interquartile range and outliers (ends of the whiskers followed by points).

Warkentin 2006; Karraker and Ruthig 2009; Ruthig 2009), as hyphae are thought to grow into the egg, perforating the egg's capsule and resulting in either death or early hatching (Touchon, Gomez-Mestre and Warkentin 2006). Interestingly, water mold had less of an effect on the timing of embryo hatching as salinity increased (Fig. 4A). This interaction is likely due to the fact that both salt and water mold are inducing eggs to hatch as early as developmentally possible. It is likely, therefore, that higher salt concentrations would not result in earlier hatching, but instead would increase egg mortality.

Because the embryonic stage is considered to be one of the most vulnerable life stages, eggs are very sensitive to environmental stressors and will often hatch early to escape potential threats (Warkentin 2011). Normal amphibian development relies on continuous transport of water across the perivitelline membrane and through the vitelline chamber containing the embryo (Krogh 1939), and increased salinity reduces the flow of water through the chamber (Gosner and Black 1957) potentially triggering early hatching. These changes to the vitelline chamber, as well as effects of salt on the jelly surrounding the

eggs, may make amphibian embryos more susceptible to infection, although this is admittedly speculative. Importantly, hatching early may result in sublethal effects on *R. sylvatica* embryos, despite not immediately causing mortality. Embryos that hatch early are less developed than those that hatch later from healthy clutches (Warkentin 1999). Less developed hatchlings are generally more vulnerable to aquatic predators (Gomez-Mestre, Touchon and Warkentin 2006) and may simply be less viable (Touchon *et al.* 2013). Increased salinization of roadside ponds may therefore cause indirect effects on amphibian populations through downstream effects after hatching.

Changes in pond salinization can prove detrimental to the survival of inhabiting organisms (Wake and Vredenburg 2008; Brühl *et al.* 2013; Gilbert 2017). This study aimed to understand how increased salinity, most likely resulting from road deicing salt runoff, interacts with water mold to influence egg mortality and hatching time of *R. sylvatica* embryos. Our results indicate that increased water conductivity likely increases water mold growth and spread in a pond. Furthermore, increasing salt concentrations had synergistic effects with the presence of water mold causing eggs to hatch early, which may in turn have downstream effects on tadpoles that survive the initial pathogen. The ramifications of premature hatching include increased predation and reduced viability (Warkentin 1999; Touchon *et al.* 2013), indicating that runoff from road deicing salt may interact with water mold in complex ways that have yet to be studied. With many amphibian species at risk around the globe, it is important that efforts be made to reduce detrimental anthropogenic influences. Alternative methods for removing ice from roadways that do not affect the salinity of nearby ponds is an important area for future research.

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