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Deicing Salts Influence Ranavirus Outbreaks in Wood Frog (Lithobates sylvaticus) Tadpoles

Sarah Jacobson
sarah.jacobson@uconn.edu

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Sarah K. Jacobson

Department of Natural Resources and the Environment, Center for Wildlife and Fisheries Conservation Center, University of Connecticut

Tracy A. G. Rittenhouse

Department of Natural Resources and the Environment, Center for Wildlife and Fisheries Conservation Center, University of Connecticut

Abstract

Ecosystems are increasingly being exposed to anthropogenic stressors that could make animals and thus populations more susceptible to disease. For example, the application of deicing salts to roads is increasing in the northeastern United States. Chronic stress that larval amphibians experience when living in vernal pools with high salinity may alter their susceptibility to ranavirus, a pathogen responsible for mass mortality events worldwide. This project quantifies the effects of road salts and ranavirus exposure on larval wood frog (*Lithobates sylvaticus*) growth and survival. Using outdoor mesocsoms, we raised wood frog tadpoles in salt treatments and then exposed them to the FV3 strain of ranavirus, with the hypothesis that individuals raised in salt treatments would have lower survival, and metamorph earlier at larger size when exposed to ranavirus than those from no salt treatments. We found that tadpoles raised in high and no salt treatments metamorphed earlier than those raised in low salt treatments ($F_{2,111}$ = 21.63, P < 0.0001). Tadpoles raised in high salt treatments had a greater mass than those raised in low and no salt treatments ($F_{2, 111}$ = 9.628, P = 0.0001). Among the tadpoles exposed to ranavirus, survival to metamorphosis was lowest in low-salt tadpoles (95% CI: 0.02 – 0.11), while survival was highest in no-salt tadpoles (95% CI: 0.29 – 0.475). In summary, exposure to road salts altered the growth and survival of larval wood frogs in non-linear ways. Our results indicated that this anthropogenic stressor is altering the response of individual tadpoles, suggesting that the disease epidemiology within populations may be altered. Additional research on the severity and frequency of outbreaks in populations is warranted.

Introduction

As the human population has grown in size, anthropogenic activities can act as a stressor to animals, wildlife populations, and their habitats. One group of animals that is especially under threat from human actions are amphibians, whose populations have been declining worldwide at a more rapid rate than all other land vertebrates (Stuart et al. 2004). Amphibian decline is a global phenomenon driven by various factors, including habitat destruction, pollution, invasive species, infectious disease, and climate change (Blaustein et al. 2011) and these factors are often specific to local areas (Grant et al. 2016). Furthermore, these factors do not simply work independently to decrease amphibian populations; more likely, multiple stressors contribute to amphibian declines and can have synergistic negative effects (Boone et al. 2007). For example, Boone et al. (2007) found that the presence of multiple factors – predation by bluegills (*Lepomis macrochirus*), competition with bullfrog tadpoles (*Lithobates catesbeina*), and exposure to ammonium nitrate fertilizer – reduced survival of larval American toads (*Anaxyrus americanus*) and spotted salamanders (*Ambystoma maculatum*) and lengthened larval periods of southern leopard frogs (*Lithobates sphenocephala*). Additionally, the contamination of aquatic habitats by pesticides has been shown to lower immune function and result in increased infection by trematode parasites in larval wood frogs (*Lithobates sylvaticus*) (Kiesecker 2002).

An environmental stressor that has garnered attention by scientists as a threat to wetland communities is the application of road salts during the winter to de-ice roads for human safety (Hintz and Relyea 2019; Schuler et al. 2018). In the United States, the quantity of deicing salt applied to roadways has increased tenfold in the last half-century (Schuler et al. 2017), and can come with serious environmental consequences for roadside wetlands and vernal pools (Karraker et al. 2008). Deicing agents are transported as solutes into habitats adjacent to roads, where they

can influence biotic and abiotic components of the environment (Karraker et al. 2008), including reducing the biomass and diversity of aquatic organisms and altering community composition by the selective loss of salt-intolerant species (Petranka and Francis 2013). Larval amphibians are particularly vulnerable to road salt because their permeable skin is used in both respiration and osmoregulation (Karraker et al. 2008). Elevated salinity in a laboratory decreased developmental rates and levels of glucose and total proteins, and increased internal osmolality in tadpoles of the European toad species, *Bufo calamita* (Gomez-Mestre et al. 2004). Deicing agents and other road contaminants can also reduce the size of larval and adult populations of amphibians in seasonal ponds near roads (Petranka and Doyle 2010). An extensive field study in upstate New York demonstrated that high concentrations of road salt reduced survival in both the embryos and larvae of wood frogs (*Lithobates sylvaticus*) and spotted salamanders (*Ambystoma maculatum*) (Karraker et al. 2008).

Although research has shown that road salt reduces amphibian survival, wood frog larvae can be found in vernal pools with rather high conductivity levels (approaching 3000 mS/m2) (Karraker et al. 2008). The more likely yet less-recognized effects of road salt on amphibians probably arise through indirect effects rather than direct lethal effects (Findlay and Kelly 2011). For example, field surveys and mesocosm experiments have shown that tadpoles grew faster and had an increased mass at metamorphosis in conditions of elevated salinity, likely because a die-off of salt-intolerant zooplankton resulted in an increase in phytoplankton, the tadpoles' primary food source (Van Meter et al. 2011; Dananay et al. 2015). Evidence suggests, however, that this positive effect on larval growth is counteracted by greater mortality in postmetamorphic juvenile frogs, possibly because of altered energy allocation, changes in behavior, or reduced immune defenses (Dananay et al. 2015).

Although high concentrations of road salts clearly have a negative effect on larval amphibians, relatively few studies have investigated whether exposure affects amphibian susceptibility to infectious diseases. Karraker and Ruthig (2009) found no interaction between road salt and pathogenic water molds on embryo survival in spotted salamanders and green frogs (*Lithobates clamitans*). In contrast, Milotic et al. (2017) found evidence that road salts increase susceptibility to infection by trematode parasites in wood frogs and northern leopard frogs (*Lithobates pipiens*) through alterations in behavior and immune response. Here we focus on ranavirus, a very lethal pathogen that was recently found to be common in CT (O'Connor et al 2016) and occurs in areas of the United States where de-icing roads is a common management practice.

Ranaviruses (Family: *Iridoviridae*) are viral pathogens responsible for mass mortality events of amphibians worldwide (Gray et al. 2009). These mass mortality events generally occur in animals undergoing metamorphosis, which is suspected to be a period of natural immune suppression (Warne et al. 2011). Previous studies have investigated whether natural and anthropogenic stressors affect host susceptibility to ranavirus, with often mixed results. For example, Reeve et al. (2013) found that chronic exposure to three natural stressors (high densities, predator cues, and low food conditions) did not make wood frog tadpoles more susceptible to ranavirus infection, as measured by the proportion of animals that became infected or died. In contrast, Pochini and Hoverman (2017) found that exposure to pesticides prior to ranavirus infection increased disease-induced mortality rates in wood frog tadpoles. Given the large number of natural and anthropogenic stressors that commonly co-occur in an amphibian habitat, it is crucial to understand how these stressors interact in order to best manage for amphibian populations.

The purpose of this experiment was to investigate the effects of road salts and ranavirus on larval wood frog (*Lithobates sylvaticus*) growth and survival. Wood frogs were chosen due to their dependence on vernal pools for breeding and high susceptibility to infection by ranavirus (Warne et al. 2011). We raised wood frog tadpoles in outdoor mesocosms under different salt treatments, and then exposed them to the FV3 strain of ranavirus. We measured survival over time, mass at metamorphosis, and time to front leg emergence and tail resorption. We predicted that as the amount of salt in the mesocosms increased, individuals exposed to ranavirus would have lower survival, higher mass, and metamorph earlier than those in the control treatments.

Methods

Salinity Treatment Set-up

The experiment took place at an outdoor mesocosm facility on University of Connecticut (UConn) property in Storrs, CT. We used a fully factorial design with three salt levels (no salt, low salt, high salt) replicated eight times for a total of 24 tanks. We filled 1000-L cattle tanks with groundwater the week of 9 April 2018, and added 1 kg of leaf litter collected from oak-dominated stands in UConn Forest to each tank. We inoculated each tank with a concentrated mixture of phyto- and zooplankton collected from natural ponds in the nearby Nathan Hale State Forest in Coventry, CT, and Merrow Meadow Park in Mansfield, CT, in order to provide a food base for the tadpoles. We covered the tanks with lids constructed from 52% shade cloth (high-density polyethylene PAK knit) that represented shade from tree canopy and prevents other amphibians and dragonflies from ovipositing in the water. The salt treatments were added to the tanks on 20 and 21 April 2018. To create the salt treatments, we obtained road salt from the Department of Transportation storage facility in Mansfield, CT, then dissolved the road salt into

15 L of water and stirred the mixture into the tanks until the concentration reached either 700 mg/L for low salt treatments, or 1400 mg/L for high salt treatments.

On 13 April 2018, we collected greater than 5 partial wood frog egg masses from one vernal pool located in Mansfield, CT. Eggs were housed in pond water within small containers in an indoor space until eggs hatched and the tadpoles reached the free-swimming stage (stage 25; Gosner 1960). We then stocked the 1000-L tanks with 50 tadpoles each on 26 April 2018. We monitored the tanks daily to check on the tadpoles and remove any adult gray tree frogs (*Hyla versicolor*) found near the tanks.

Ranavirus Exposure

For the ranavirus exposure tests, we used nine 189-L (50-gal) Rubbermaid tanks laid out in blocks of three in the same field as the 1000-L tanks. The ranavirus treatments were replicated twice for each salt treatment, however, control tanks were not replicated due to the limited number of available tanks. We filled the smaller tanks with groundwater on 4 – 5 June 2018. The water was continually pumped through ultraviolet sterilizers to inactivate pathogens within the water column. As we filled the smaller tanks, we drained the 1000-L tanks and removed the tadpoles using a hand net. We brought the tadpoles to the indoor animal care facility, and staged tadpoles under a dissecting microscope. We then marked half with fluorescent visible implant elastomer (Northwest Marine Technology, Inc) injected under the skin on the dorsal area of the body. We performed this step while the tadpoles were under anesthesia (0.025 g/mL MS-222; Brunner et al. 2017). Tadpoles from different treatments were batch marked with different colored tags; yellow for control, orange for low salt, and red for high salt. We then stocked the tanks with 60 tadpoles – 30 marked and 30 unmarked – each. All tadpoles were held in

freshwater from this part of the experiment onward. We fed the tadpoles ground-up algae wafers daily.

To infect populations with ranavirus, we selected 9 tadpoles that were raised in the control mesocosms and marked these individuals with green VIE tags. We then injected 6 tadpoles with the 100ml of 10^4 plague-forming units of FV3 strain of ranavirus prepared by Dr. Jesse Brunner (Washington State University) and 3 tadpoles with 100ml of 2% FBS-HEM as controls. The tadpoles were held in the indoor facility overnight to ensure no immediate mortality resulting from handling stress. We introduced one infected or control tadpole into each of the 9 tanks on 7 June 2018. We checked the tanks daily for metamorphs, defined as when the front legs emerged (stage 42; Gosner 1960), and dead tadpoles. The dead tadpoles were removed using hand nets and then preserved in 95% ethanol. We housed the metamorphs in a small container until tail resorption (stage 46, Gosner 1960), upon which they were massed and then euthanized using an overdose of MS-222. We then placed each individual in separate Whirl-Pak (Nasco) bags containing 95% ethanol. Data collection ended on 2 July 2018, when all of the tadpoles were either dead or metamorphed out of the tanks.

In September 2018, we sent three preserved specimens from each tank, as well as three specimens collected from the source wetland on 15 June 2018, to the Amphibian Disease Diagnostic Laboratory at Washington State University for ranavirus testing. Tissue samples from each specimen were screened for ranavirus DNA with a quantitative TaqMan real time polymerase chain reaction (qPCR) assay specific to the major capsid gene of all known ranaviruses except for one divergent group (DFV, GV6, LMBV; Stillwel et al. 2018). DNA was extracted with the Qiagen DNEasy Blood and Tissue kit and then 5 µL of DNA template was included in triplicate 20 µL reactions run for 45 cycles under standard conditions on a

StepOnePlus thermocycler (Applied Biosystems). A serial dilute of a gBlock (Integrated DNA Technologies) DNA fragment that includes the target sequence served as the standard for quantitation and a no-template control as a negative control on each plate. Samples were scored positive if at least two of the three wells had clear amplification and negative if zero of three wells had amplification. If there was amplification in just one of the three wells, the sample was re-run and considered positive if greater than 1 well was positive and negative if not.

Data Analysis

We performed a survival analysis using the survival and survminer packages in Program R, separating survival into two categories: survival of the tadpoles up to front leg emergence (Gosner Stage 42), and survival from when the tadpoles were exposed to ranavirus to complete metamorphosis (Gosner Stage 46). We created a Kaplan – Meyer survival curve for each treatment, and compared the survival estimates of the different treatments using the cumulative survival values at day 25 after ranavirus was introduced into the tanks.

We analyzed the effect of road salt, ranavirus, and mark on time to front leg emergence, time to tail resorption, and mass at metamorphosis, by creating a linear model and performing a three-way ANOVA for each response variable in Program R (R Core Team 2019). We additionally performed a one-way ANOVA to analyze the effect of the different salt treatments on the Gosner stage of the tadpoles when we transferred them from the 1000-L tanks to the 189-L tanks. We tested for model assumptions of normality using a Q-Q plot and equal variance by plotting residuals vs fitted values. We reported in the figures the estimated marginal means, also known as least-squared means.

Results

Summary of mortalities

We exposed 366 (122 from each salt treatment) of the 549 tadpoles to ranavirus. Of the tadpoles raised in the no-salt treatment, 24 (19.7%) tadpoles died before reaching front leg emergence and 45 (37.0%) died before completing metamorphosis. In the low-salt treatments, 48 (39.3%) tadpoles died before reaching front leg emergence and 65 (53.3%) died before completing metamorphosis. In the high-salt treatments, 17 (14.0%) tadpoles died before reaching front leg emergence and 73 (59.8%) died before completing metamorphosis. Mortalities were also observed among the 183 tadpoles (61 in each salt treatment) in the control tanks. In the no-salt treatment, 1 (1.6%) tadpole died before reaching front leg emergence and 49 (80.3%) died before completing metamorphosis. In the low-salt treatment, 19 (31.1%) tadpoles died before reaching front leg emergence, and 30 (49.2%) died before completing metamorphosis. Of the tadpoles raised in the high-salt treatment, 10 (16.4%) died before reaching front leg emergence and 29 (47.5%) died before completing metamorphosis. Of the 118 total individuals that survived to complete metamorphosis, 70 (59.3%) were marked. We were unable to identify 9 individuals as marked or unmarked because carcasses decomposed and were pulled into the filter. Because of a pump malfunction on 17 June 2018, we excluded a single no salt – ranavirus tank (12 individuals) after day 10 of the experiment from data analysis.

Results from Kaplan-Meyer Survival Analysis

Survival of the tadpoles to front leg emergence was high (>50%) across all treatments (Table 1; Figure 1; Figure 3). For both the ranavirus and control tanks, tadpoles that were raised in the low salt treatments had the lowest survival, being 0.597 (95% CI: 0.515 – 0.692) for the ranavirus treatments and 0.683 (95% CI: 0.575 – 0.812) for the control. Survival for animals

raised in the no salt treatments was higher in the control (0.98, 95% CI: 0.95 – 1.00) than in the ranavirus treatments (0.78, 95% CI: 0.71 – 0.86), while survival for individuals raised in the high salt treatments were relatively the same (0.84, 95% CI: 0.76 – 0.94 for ranavirus; 0.855, 95% CI: 0.79 - 0.92 for control).

Survival from the beginning of the ranavirus experiment to tail resorption was low across all treatments, not reaching above 50% even in the control tanks (Table 1; Figure 2; Figure 3). For the ranavirus tanks, tadpoles that were raised in the low salt treatments had the lowest survival of 0.05 (95% CI: 0.02 – 0.11), while animals raised in the no salt treatments had the highest survival of 0.37 (95% CI: 0.29 – 0.475). Tadpoles in the control tanks displayed a different pattern, where animals raised in the no salt treatments had the lowest survival at 0.153 (95% CI: 0.084 – 0.28) and individuals raised in the high salt treatments had the highest survival at 0.38 (95% CI: 0.28 – 0.52).

Effects on Growth

When staging the tadpoles, we found that they were all at a late stage in development (stages 36-40; Gosner 1960). Additionally, we observed that the no and high salt tadpoles were, on average, 2 to 3 Gosner stages higher than the low salt tadpoles (Figure 4). From the one-way ANOVA, we found that the relationship between salt and stage was significant, indicating that salt had an impact on development at the beginning of the ranavirus exposure tests ($F_{2, 368} = 64.063$, $P < 0.001$) (Table 2).

We found that the linear models that we created for mass at metamorphosis, time to leg emergence, and time to tail resorption met model assumptions of normality and equal variance. Both ranavirus and road salt had a significant effect on time to leg emergence ($F_{1, 402} = 98.07$, $P < 0.0001$; $F_{2, 402} = 40.37$, $P < 0.0001$) (Table 3). When exposed to ranavirus, individuals reached

Gosner stage 42 roughly 3 days faster than those in the control tanks (Table 1; Figure 5). Additionally, tadpoles raised in the high salt and no salt treatments reached leg emergence on average 4 days faster than individuals raised the low salt tanks (Table 1; Figure 5), indicating a non-linear relationship. Despite these effects, we found no significant interaction between ranavirus and salt on time to leg emergence ($F_{2, 402} = 1.739$, $P = 0.177$).

Both road salt and ranavirus had a significant effect on time to tail resorption ($F_{1, 111} = 87.32$, $P < 0.0001$; $F_{2,111} = 21.63$, $P < 0.0001$) (Table 3). Additionally, we found evidence of an interaction between road salt and ranavirus on time to tail resorption ($F_{2,111} = 5.371$, $P = 0.0059$). When exposed to ranavirus across all salt treatments, tadpoles completed metamorphosis on average 5 days faster than those in the control tanks (Table 1). Of the tadpoles exposed to ranavirus, those raised in no salt and high salt treatments completed metamorphosis sooner than individuals raised in the low salt treatments. In the control tanks, however, tadpoles raised in the high salt treatment completed metamorphosis the earliest, with tadpoles reaching tail resorption later when raised in the low and no salt treatments (Table 1; Figure 6). The difference between time to tail resorption in the ranavirus and control tanks was magnified for individuals raised in the no salt treatment, where tadpoles exposed to ranavirus completed metamorphosis roughly 9 days faster than their control counterparts (Table 1; Figure 6).

Ranavirus and road salts had significant effects on the tadpoles' mass at complete metamorphosis ($F_{2, 111} = 9.628$, $P = 0.0001$; $F_{1, 111} = 9.835$, $P = 0.0022$) (Table 3). Tadpoles raised in the high salt treatment had a higher mass at tail resorption than tadpoles raised in the low and no salt treatments, and individuals in the ranavirus-treated tanks reached complete metamorphosis at a larger mass than those in the control tanks () (Table 1; Figure 7). We found no evidence of a significant interaction between ranavirus and salt on mass ($F_{2, 111} = 0.222$, $P = 0.770$). Additionally,

among all of the ANOVA tests, we found no evidence of an interaction between mark and time to front leg emergence, time to tail resorption, and mass at metamorphosis (Table 1; Table 3).

Ranavirus Testing Results

All of the specimens sent to the Amphibian Disease Diagnostic Laboratory tested positive for ranavirus, but the mean copy of the MCP gene was 10^5 or 10^6 times greater in the ranavirus treatments than in the control treatments (Table 4). Additionally, ethanol from the Whirl-Paks had spilled in the Ziploc bag containing the specimens. The spilled ethanol was tested and came back positive for trace amounts of ranavirus, making it possible that the trace amounts of ranavirus (>5 copies of the MCP gene) detected in some of the specimens may have come from the spilled ethanol.

Discussion

Overview

Understanding how different stressors interact and impact amphibian populations is vital for addressing and managing against population declines. In this experiment, we used outdoor mesocosms to test how wood frog tadpoles raised in different salt treatments would respond to exposure to ranavirus. From our results, we have two interesting findings: 1) Salinity had a non-linear effect on growth, and 2) Salinity had an indirect effect on tadpole response to ranavirus, with the most negative affects occurring at low salinity.

Non-Linear Effects of Salt on Development

Our prediction that tadpoles raised in the high salt treatment would have a higher mass at metamorphosis was supported by our results and aligns with results in literature (Van Meter et al. 2011; Dananay et al. 2015). In contrast, development, measured by time to front leg emergence and time to tail resorption was roughly the same between the no and high salt treatments, with

individuals in the low salt treatment taking longer to metamorphose than individuals in the no and high salt treatment. When staging the tadpoles prior to transferring them to the ranavirus treatment tanks, we noticed that the low salt tadpoles were, on average, at an earlier stage of development than the no and high salt tadpoles, which could explain the difference in the times to metamorphosis. Several studies have found that higher salt concentrations (up to 900 mg/L) caused wood frog larvae to be larger but less developed than larvae in lower salt concentrations (Dananay et al. 2015; Hall et al. 2017). In contrast, Van Meter et al. (2011) found that elevated chloride (645 mg/L) in mesocosms decreased the number of days it took for gray treefrog (*Hyla versicolor*) tadpoles to reach metamorphosis. These differing results indicate that responses to road salts likely differ among species and concentration of road salt used. It is possible that at the high salt concentration we used (1400 mg/L), the tadpoles were stressed enough to increase their development and complete metamorphosis sooner than those raised in the low salt concentrations. 1400 mg/L is on the high end in terms of concentrations larval wood frogs can tolerate; acute toxicity tests performed by Collins and Russel (2009) found that the lowest median lethal chloride concentration for wood frogs was 1721.4 mg/L.

Indirect Effects of Salt on Tadpole Response to Ranavirus Exposure

Within all salt treatments, exposure to ranavirus slowed development and increased mass at metamorphosis. These findings are supported by previous research, which found that infection by ranavirus accelerated developmental rates, likely as a stress response to the disease (Warne et al. 2011). Likewise, two studies (Forson and Storfer 2006; Kerby and Storfer 2011) investigating the effects of the ATV ranavirus on larval tiger salamanders (*Ambystoma tigrinum*) found that the virus caused a slight, but significant increase in mass at metamorphosis. The slight increase

in mass has been attributed to edema, a common symptom of ranavirus (Forson and Storfer 2006).

Among the tadpoles that were exposed to ranavirus, survival to metamorphosis was lowest in individuals that were reared in the low salt treatments, while survival was highest in individuals that were reared in the no salt treatments. This result provides evidence that road salts indirectly impact survival of larval wood frogs when exposed to ranavirus. It should be noted that survival for individuals raised in the high salt treatments was higher than those in the low salt treatments, which we assume is due to the difference in developmental stage between the two; being at a later stage of development, the high salt tadpoles were able to complete metamorphosis before succumbing to the virus. If we had exposed the tadpoles to ranavirus at an earlier stage, the results likely would been different (Haislip et al. 2011), as more tadpoles would have likely succumbed to the virus before metamorphosing out of the tanks.

Additional Considerations

We found no significant effects of VIE tagging on survival, time to front leg emergence and tail resorption, and mass at metamorphosis, upholding the assumption that this form of marking has no effect on tadpole growth and survival. This assumption is crucial, as marking is an important tool for studying populations and distinguishing cohorts in experiments (Bainbridge et al. 2015; Grant 2008). This finding indicates that VIE tagging can be used safely when analyzing effects of ranaviruses on amphibians in future projects.

We also observed high mortalities in the control tanks, the majority of which were post-front leg emergence when the metamorphs were out of the tanks and in small containers in the indoor facility. The ultimate cause of the mortalities is unknown. As metamorphs from both the control and the ranavirus tanks were housed in the same building, we suspect that there could

have been some cross-contamination, as ranavirus can be spread easily through infected water (Gray et al. 2018). When we sent specimens to the Amphibian Disease Diagnostic Laboratory, they were all tested positive for ranavirus, however we observed a great difference in the mean copy of the MCP gene between the control and ranavirus tanks. This result could be explained by the spilled ethanol during the shipment and/or the tadpoles carried trace amounts of the virus from their source pond. Additionally, amphibians undergoing metamorphosis experience immunosuppression, making them vulnerable to outside stressors (Gray et al. 2009). This project included transferring late-stage tadpoles from large 1000L mesocosms into smaller 50-gal tanks for the ranavirus exposure tests. These smaller mesocosms were needed to contain the virus and enhance our ability to observe individual tadpoles. However, mortality in the control treatments suggests that the transferring process may have unintentionally added an important source of stress to the tadpoles. Transferring tadpoles at an earlier stage of development, or exposing the tadpoles to ranvirus in the same tank they were raised in, could alleviate this possibility in future studies. We advise future experimenters to take care when transferring metamorphs in disease studies, and to install appropriate measures to prevent cross-contamination.

Conclusion

In this project, we sought to understand how road salt and ranavirus, two stressors affecting amphibians in the United States, impact larval wood frog growth and survival. Our results indicate that road salt and ranavirus have significant effects on the growth and survival of larval wood frogs, but the interactions between the two stressors remain unclear and likely depend on the developmental stage and other physiological and environmental factors. Topics of further investigation could include exposing individuals to ranavirus and road salt at different developmental stages, as well as assessing the effects post-metamorphosis. This study indicates

that road salt is an anthropogenic stressor that alters disease epidemiology, which could lead to changes in the severity and frequency of ranavirus outbreaks.

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Tables and Figures

Table 1. Least squared means for mass at metamorphosis, time to front leg emergence (days after ranavirus introduction), time to tail resorption (days after ranavirus introduction), and estimated K-P survival amongst categories in experiment.

Treatment	No. Tadpoles	Mass (g)	Time (days) to Front Leg Emergence	Time (days) to Tail Resorption	K-P Survival to Front Leg Emergence	K-P Survival to Tail Resorption
NoSaltRanavirus	122	0.454 ± 0.033	3.78 ± 0.73	6.34 ± 0.97	0.782	0.373
LowSaltRanavirus	122	0.447 ± 0.087	7.46 ± 0.80	11.83 ± 2.54	0.597	0.050
HighSaltRanavirus	122	0.539 ± 0.041	4.61 ± 0.67	6.70 ± 1.20	0.855	0.231
NoSaltControl	61	0.366 ± 0.071	7.83 ± 0.88	15.33 ± 2.07	0.983	0.153
LowSaltControl	61	0.374 ± 0.064	11.32 ± 1.05	14.82 ± 1.88	0.683	0.183
HighSaltControl	61	0.483 ± 0.043	7.23 ± 0.92	11.92 ± 1.27	0.841	0.381
Marked	270	0.479 ± 0.015	6.40 ± 0.32	9.29 ± 0.66	0.767	0.270
Unmarked	270	0.444 ± 0.014	6.40 ± 0.26	9.33 ± 0.56	0.799	0.180

Table 2. One-way ANOVA for Gosner Stage at start of ranavirus trial.

Source	df	Sum Sq	Mean Sq	F value	P value
Salt	2	408.44	204.22	64.063	1.34E-24
Residuals	368	1173.1	3.1878		

Table 3. Three-way ANOVA for Mass, Time to Leg Emergence, and Time to Tail Resorption.

	Source	df	Sum Sq	Mean Sq	F value	P value
Mass	Salt	2	0.2216	0.1108	9.6283	0.00014
	Ranavirus	1	0.1132	0.1132	9.8346	0.00219
	Mark	1	0.0061	0.0061	0.5287	0.4687
	Salt:Rana	2	0.0060	0.0030	0.2623	0.7698
	Residuals	111	1.2776	0.0115		
Time to Leg Emergence	Salt	2	946.75	473.38	40.368	1.06E-16
	Ranavirus	1	1150.0	1150.0	98.065	7.84E-21
	Mark	1	2.4303	2.4303	0.2072	0.6492
	Salt:Rana	2	40.793	20.397	1.7393	0.1770
	Residuals	402	4714.1	11.727		
Time to Tail Resorption	Salt	2	429.67	214.84	21.628	1.17E-08
	Ranavirus	1	867.35	867.35	87.320	1.16E-15
	Mark	1	5.0907	5.0907	0.5125	0.47556
	Salt:Rana	2	106.71	53.355	5.3714	0.00593
	Residuals	111	1102.6	9.9331		

Table 4. Results from ranavirus testing for each treatment.

Treatment	No. Tested	Mean Copy
NoSaltRanavirus	6	5.07E+05
NoSaltControl	3	3.41
LowSaltRanavirus	6	7.72E+06
LowSaltControl	3	3.92
HighSaltRanavirus	6	4.35E+06
HighSaltControl	3	3.97
Source Pond	3	0.47
Spilled Ethanol	N/A	0.72

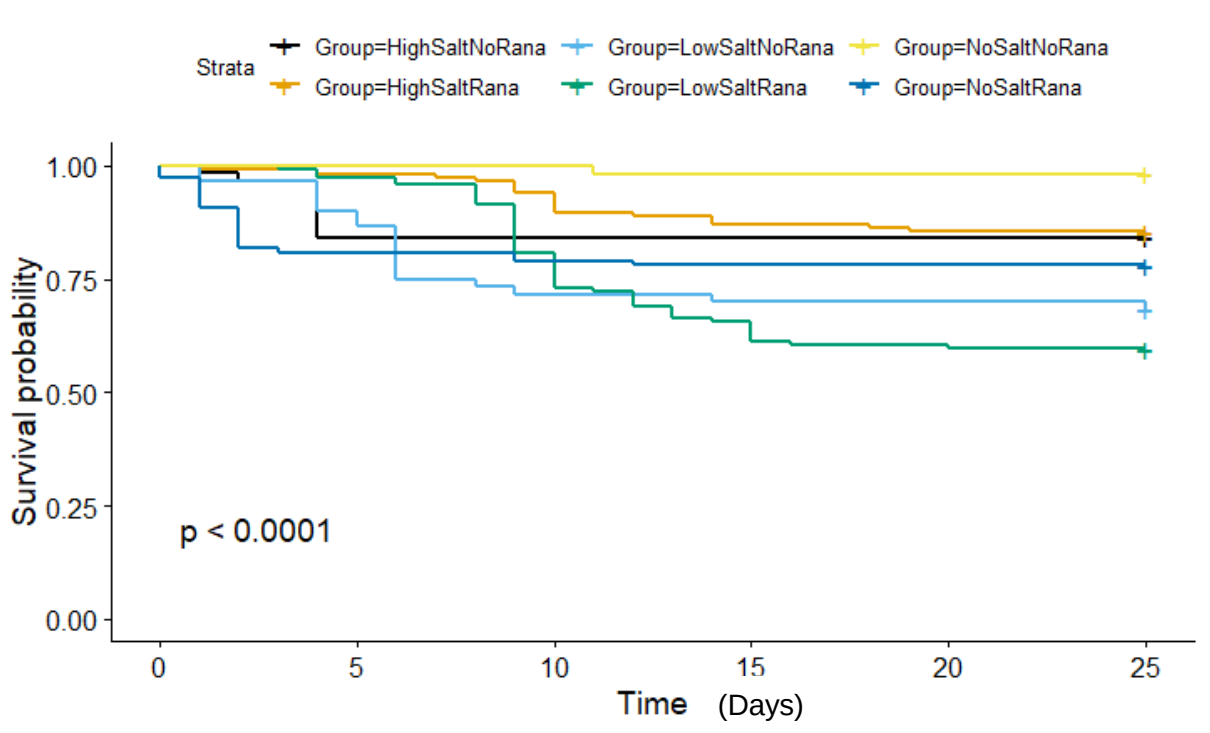

Figure 1. Kaplan – Meyer cumulative survival curve to front leg emergence.

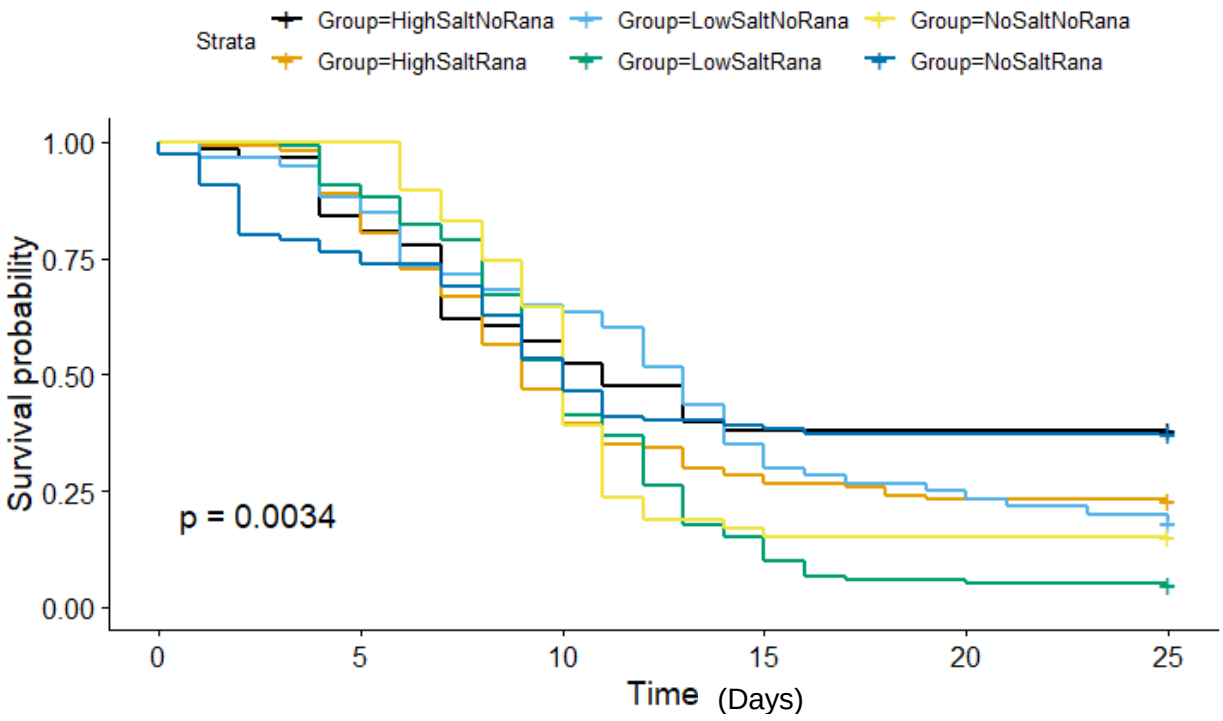

Figure 2. Kaplan - Meyer cumulative survival curve to tail resorption.

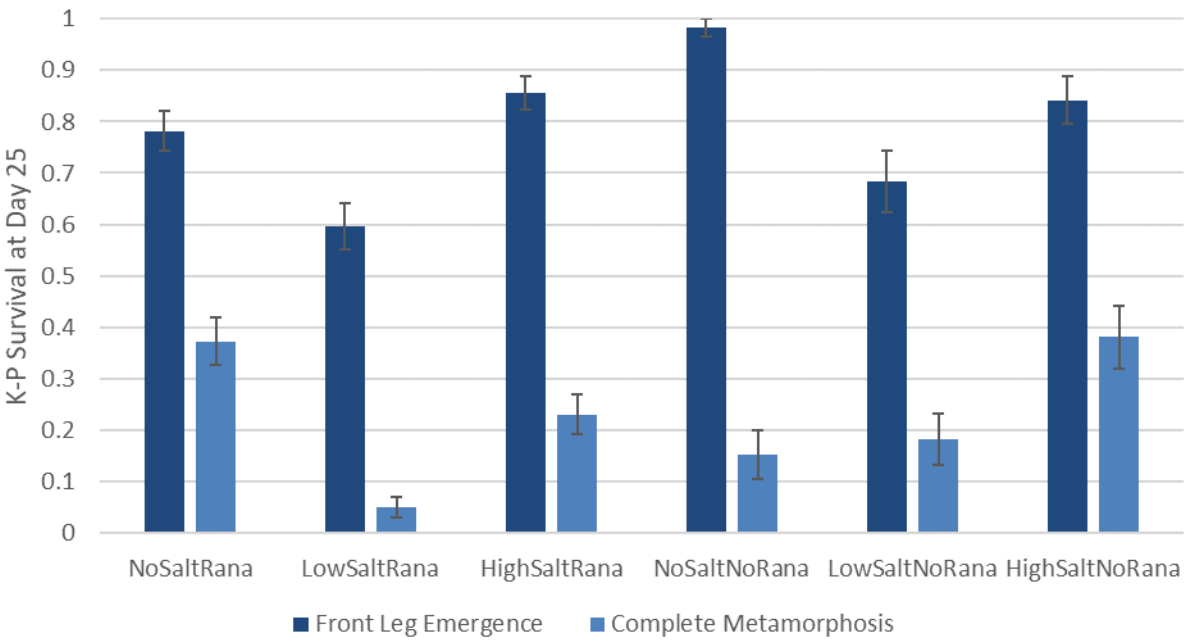

Figure 3. Kaplan-Meyer cumulative survival from ranavirus introduction at day 1 to day 25.

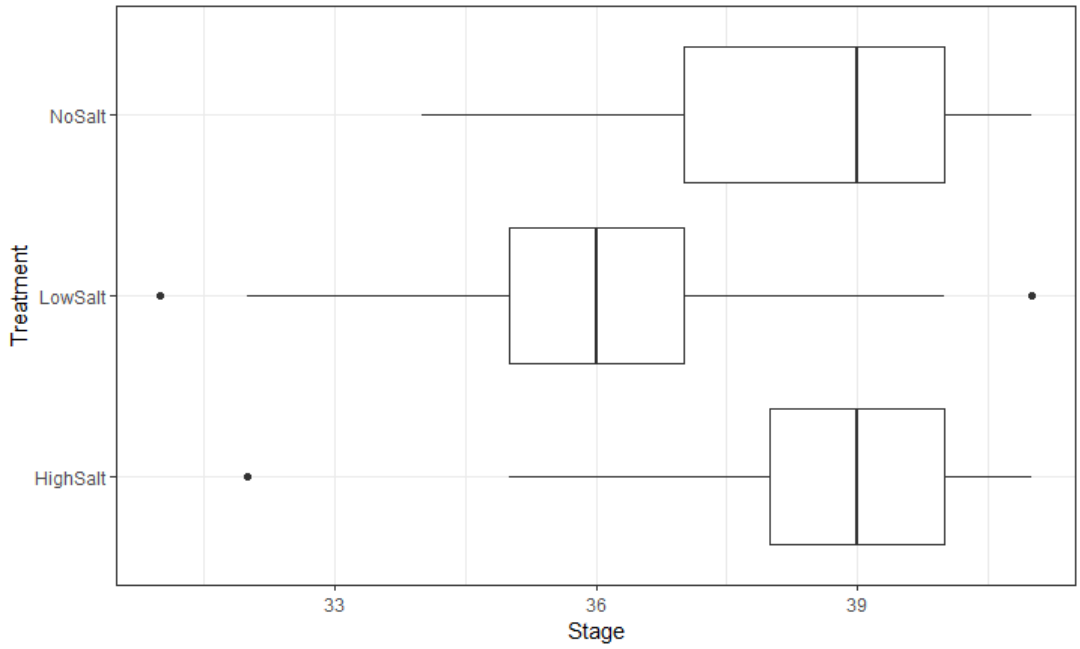

Figure 4. Variation in Gosner Stage between the different salt treatments.

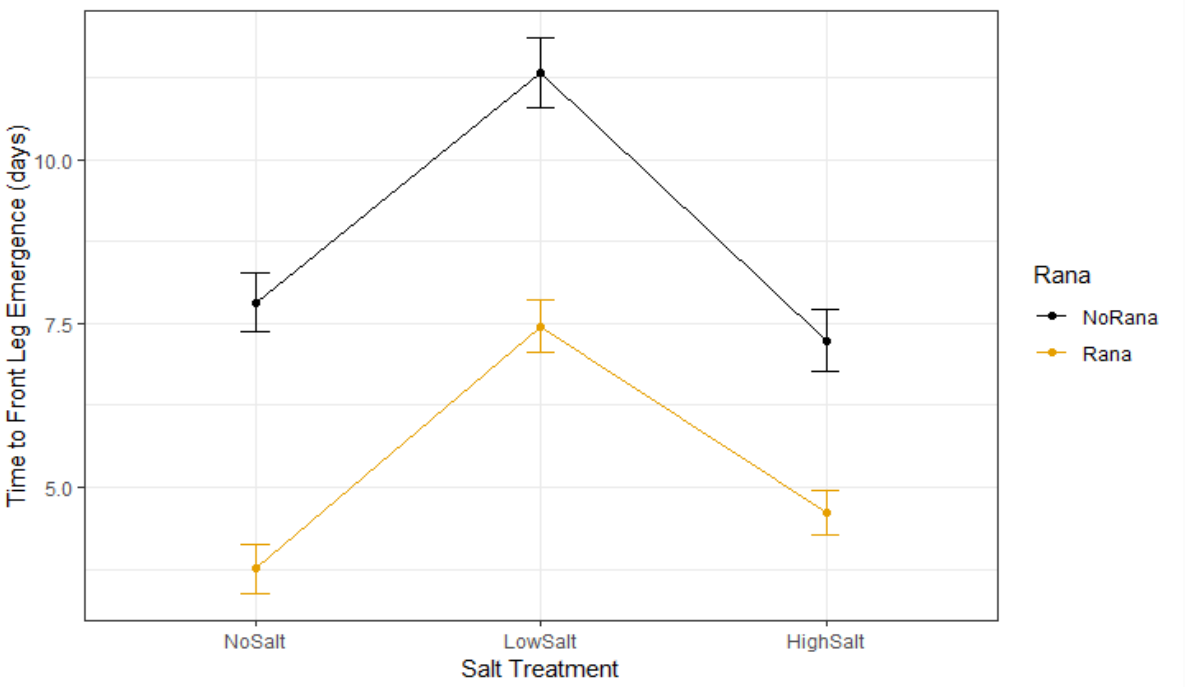

Figure 5. Least squared means for time to leg emergence.

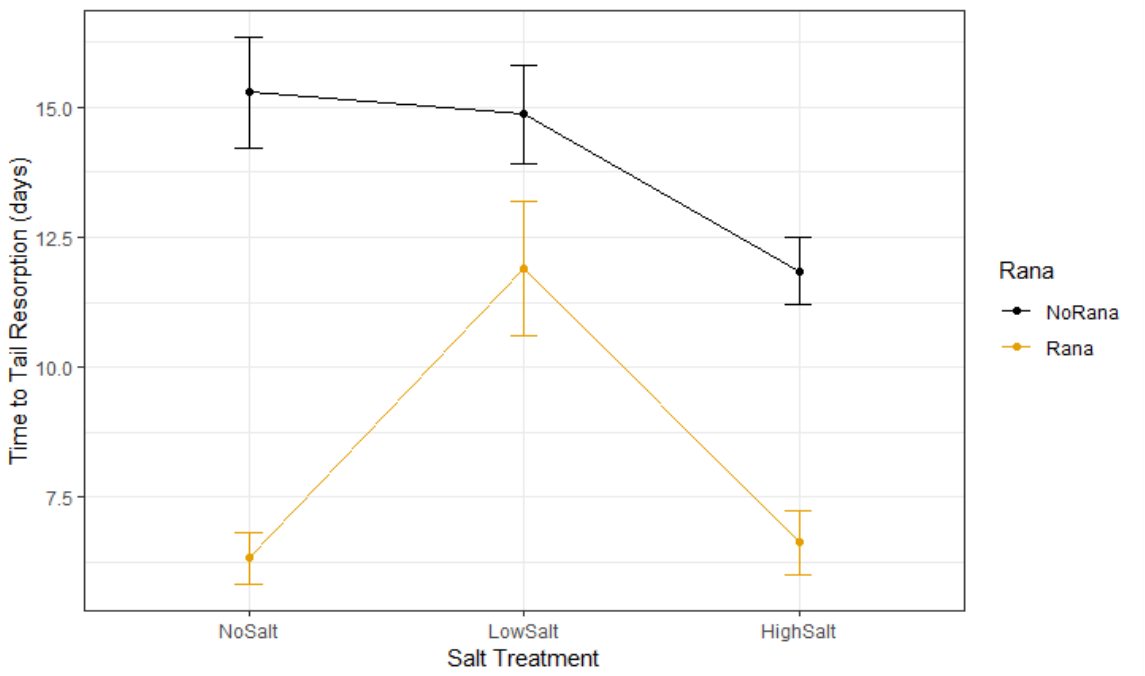

Figure 6. Least squared means for time to tail resorption.

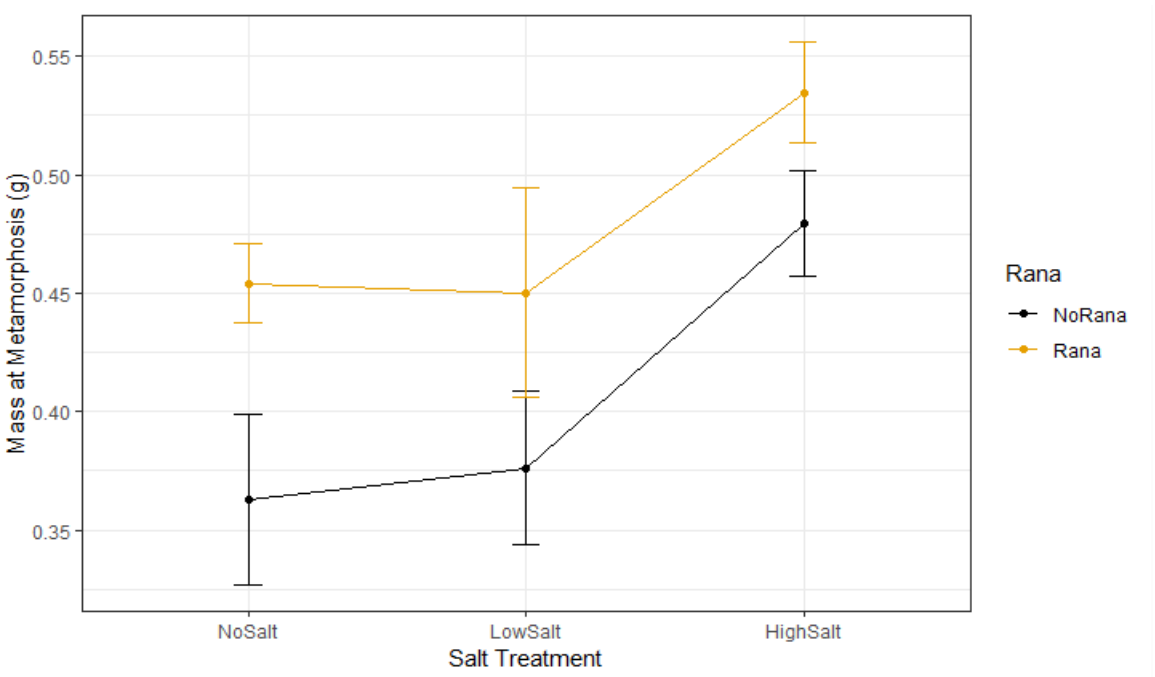

Figure 7. Least squared means for mass at metamorphosis.