

Salinity Conditions during the Larval Life Stage Affect Terrestrial Habitat Choice in Juvenile Wood Frogs (*Lithobates sylvaticus*)

ZACHARY T. VEGSO,^{1,2,4} ANILA A. KALONIA,^{1,3} SKYLER STEVENS,¹ AND TRACY A. G. RITTENHOUSE¹

¹ Department of Natural Resources and the Environment, University of Connecticut, Storrs, Connecticut, 06269, USA

² Department of Biology, Appalachian State University, Boone, North Carolina, 28607, USA

³ Department of Biological Sciences, Humboldt State University, Arcata, California, 95521, USA

ABSTRACT.—Anthropogenic salinization is a pervasive pollutant in much of the northeastern United States because of the widespread use of chemical deicing agents on roads. Although studies have examined the physiological effects of salinization on amphibians across life stages, behavioral responses to salinization of habitats are less studied. In this study, we experimentally test how salinity and temperature conditions experienced as larvae affect behavioral and physiological responses as juveniles. We first experimentally test whether juvenile Wood Frogs (*Lithobates sylvaticus*) can detect and avoid road salt in terrestrial soils and whether this avoidance behavior differs depending on temperature and salinity conditions in which individuals were raised as larvae. We also experimentally test whether temperature and salinity conditions experienced as larvae affect desiccation rates in juvenile Wood Frogs. We found a significant correlation between larval salinity conditions and choice of soil, with frogs raised in high salt aquatic conditions spending the majority of time on high salinity soils and frogs raised in low salt aquatic conditions spending the majority of time on low salinity soils. This behavioral response was muted in frogs raised in elevated temperature conditions. We were unable to detect a correlation between larval treatment and desiccation rate. Our experiments demonstrate that Wood Frogs can detect and respond to salinity levels in terrestrial habitats and that this juvenile response depends on environmental conditions experienced as larvae.

While amphibian declines are a global phenomenon (Stuart et al., 2004; Wake and Vredenburg, 2008), stressors causing changes in population trends are localized and can vary on regional scales (Grant et al., 2016). The impacts of exurban development threaten many amphibian species, both through the destruction of breeding and nonbreeding habitat and the lethal and sublethal effects of pollutants (Baldwin and deMaynadier, 2009). Salinization is growing conservation concern that is linked to urbanization and road transportation of people and goods (Hintz and Relyea, 2019). In the United States, where roads and highways are estimated to impact ecological processes on 15–20% of the country's land area (Forman and Alexander, 1998), the effects of roadways provide one such localized threat to amphibians of the region.

Worldwide, the presence of roadways exposes amphibians and other wildlife to a wide range of hazards and ecological impacts. Consequences include road mortality; habitat fragmentation; road avoidance due to traffic noise; erosion; sediment loading of streams; and chemical pollution, including heavy metals and salts that run off into nearby waterbodies (Forman and Alexander, 1998; Trombulak and Frissell, 2000; Tennesen et al., 2014; Hall et al., 2017). Furthermore, salinization of forested wetlands changes ion exchange, including carbon and nitrogen fluxes from wetlands (Ardón et al., 2018). In the northeastern United States and other high-latitude regions, anthropogenic salinization has become a pervasive pollutant due to the widespread use of chemical deicing agents (Jackson and Jobbágy, 2005; Kaushal et al., 2005; Karraker et al., 2008; Findlay and Kelly, 2011; Hall et al., 2017). In some urban and suburban streams, peak chloride concentrations can reach up to 25% of that of seawater, with elevated concentrations persisting into the spring, summer, and autumn (Kaushal et al., 2005). These chloride levels have led to increasing concerns for maintaining healthy aquatic systems

and drinking water supplies (Huling and Hollocher, 1972; Kaushal et al., 2005; Daley et al., 2009).

Recent research indicates that the effects of salinization extend into terrestrial environments. For example, salt concentrations of up to 300 g/m² can be found in surface soils within 10 m of salinized roadways, with elevated salt concentrations continuing up to 100 m from roadways (Lundmark and Olafsson, 2007; Green et al., 2008; Findlay and Kelly, 2011). Salts are transported more slowly through soils and shallow groundwater than surface water, and there is increasing evidence that salt is retained in terrestrial environments long after winter application (Bastviken et al., 2006; Kelly et al., 2008; Robinson et al., 2017). The impacts of road salinization on amphibians are not limited to aquatic environments; they also may have extended repercussions across terrestrial environments.

Terrestrial amphibians must respond to environmental cues to continue functional ionic and osmotic regulation. Amphibian skin is highly vascularized, serving as a respiratory and osmoregulatory surface. However, this condition results in rapid rates of evaporative water loss in most species (Hillyard, 1999). Amphibian skin has historically been understood as being freely permeable to evaporative water loss; however, some arboreal species are now known to possess adaptations to reduce evaporative water loss (Toledo and Jared, 1993). In ranids, resistance to evaporative water loss is predictably low (Spotila and Berman, 1976), with Adolph (1932) observing that skinned Northern Leopard Frogs (*Lithobates pipiens*) lose water at the same rate as living frogs.

The movement of ions across amphibian skin takes place through a variety of transport mechanisms. Na⁺ enters the cells passively through epithelial sodium channels (ENaCs) and is regulated via the Na⁺-K⁺ ATPase pump and K_{ATP} channels (Jared and Rao, 2017). Flask-shaped mitochondria-rich (MR) cells also intersperse the epithelium. MR cells are thought to be important in the regulation of pH and Cl⁻ fluxes, particularly at low external salinities (Budtz et al., 1995; Hillyard, 1999; Jared and Rao, 2017). Mechanisms of ion transport in larval frogs differ from those of adults. In American Bullfrogs (*Lithobates*

⁴ Corresponding Author. E-mail: vegsozt@appstate.edu
DOI: 10.1670/20-123

catesbeianus), adult mechanisms of Na^+ and Cl^- transport do not appear in the skin until Gosner stage 42. Before this stage, Na^+ and Cl^- transport takes place in the gill chambers (Alvarado and Moody, 1970).

Temperature also influences ionic and osmotic regulation in amphibians. Adult amphibians respond to changes in temperature by altering their osmoregulatory “set point” (Brown et al., 1986; Jørgensen, 1991). When frogs and tadpoles adapted to warm temperatures are exposed to cold temperatures (e.g., during hibernation or overwintering as larvae), body water content increases, resulting in an increase of mass (Bradford, 1984; Jørgensen, 1991). Under these conditions, internal Na^+ concentration is retained in adults; however, Bradford (1984) found that larvae lose much of their internal Na^+ within 1 mo of exposure. Na^+ concentration is restored within 7 mo, although not completely in some species. Brown et al. (1986) found that when larvae and postmetamorphic American Bullfrogs adapted to cold temperatures were exposed to warm water (11 and 18°C), the animals lost from 7 to 10% of their body mass due to a reduction of body water content; extracellular Na^+ concentration appeared to increase 28 and 21%, respectively. These responses appear to be hormonally regulated, because tadpoles treated with ovine prolactin or ovine growth hormone did not experience weight loss or the increase in Na^+ concentrations.

In addition to anthropogenic salinization, wetlands in northern climates and worldwide are likely to experience increased temperatures because of climate change. Temperature is a strong factor in regulating amphibian physiology, most notably by its influence of thyroxine production (Smith-Gill and Berven, 1979; Denver, 1997; Bellakhal et al., 2014). The presence of multiple physiological stressors adds a layer of complexity to studying ecological trends. Although several studies have examined the effects of elevated temperature (Köhler et al., 2011; Duarte et al., 2012; Ruthsatz et al., 2018) and salinity (Karraker et al., 2008; Denoël et al., 2010; Dananay et al., 2015; Hall et al., 2017; Milotic et al., 2017) on amphibians independently, few have studied the combined interaction between these two key stressors (Green et al., 2019).

Previous studies have examined the physiological and demographic effects of anthropogenic salinization on amphibians across life stages, finding evidence for increased larval growth rate, reduced embryonic and hatchling survival, reduced postmetamorphic survival, increased susceptibility to parasitism and disease, and increased water retention in adult males (Karraker et al., 2008; Dananay et al., 2015; Hall et al., 2017, 2020; Milotic et al., 2017). Other studies have used common garden experiments to examine coastal amphibian populations, finding that although both populations are negatively affected by elevated salinity levels, coastal populations possess a greater tolerance to salt than their inland counterparts (Hopkins et al., 2016; Albecker and McCoy, 2017). Comparatively few studies have researched the behavioral impacts of anthropogenic salinization on amphibians, although Denoël et al. (2010) found that larval Common Frogs (*Rana temporaria*) showed reduced speed and movement when exposed to elevated salinity conditions. In addition, behavioral studies focused on salinity are limited to a single life stage (Denoël et al., 2010). Few studies, if any, have examined how salt exposure during one stage carries over to affect subsequent stages. Carryover effects are of particular importance given that postmetamorphic amphibians are likely to encounter elevated salinity in the soils surrounding roadside wetlands (Lundmark and Olafsson, 2007; Findlay and Kelly, 2011). Here, we focus on

carryover effects from the larval to juvenile life stage and on behavioral responses to increased salts, because terrestrial habitat selection results from not only survival in terrestrial habitats (Rittenhouse et al., 2008) but also the behavioral choices made by frogs when migrating from wetlands to nonbreeding habitats (Rittenhouse et al., 2004; Patrick et al., 2008; Rittenhouse and Semlitsch, 2009).

We first conduct a behavioral choice experiment on whether juvenile Wood Frogs (*Lithobates sylvaticus*) can detect and avoid salt in terrestrial soils. This experiment also tests whether this avoidance behavior differs depending on the temperature and salinity conditions in which individuals were raised as larvae. We then experimentally test whether temperature and salinity conditions experienced as larvae affect desiccation rates in juvenile Wood Frogs. We used this frog as our study species because it is an early breeding anuran common throughout much of the higher latitude regions of North America and it is thus exposed to high levels of salinization from road runoff in early spring.

We hypothesize that conditions as larvae have a significant effect on choice of soil as juveniles. We predict that frogs raised in high salt conditions select high salinity soils and that frogs raised in low salt conditions select low salinity soils. We do not expect temperature conditions alone to have a significant effect on juvenile choice of soil; however, there may be an interaction between larval salinity and temperature conditions that influences juvenile choice of soil. We also hypothesize that conditions as larvae have a significant effect on desiccation rates as juveniles. We predict that individuals raised in high salt conditions have a lower dehydration rate than those raised in low salt conditions. We do not expect temperature conditions as larvae to be a significant factor in desiccation rates as juveniles.

MATERIALS AND METHODS

Egg masses were collected from six local ponds near Mansfield, Connecticut. We raised larval Wood Frogs, as part of an ongoing *Ranavirus* project, in 50-gal (189-L) mesocosms under four treatments based on temperature and salinity. Our two temperature treatments were ambient (natural, unmanipulated water temperature) and elevated (raised 3°C above ambient). Our two salinity treatments were high salt (1,900–2,000 $\mu\text{S}/\text{cm}$) and low salt (52–102 $\mu\text{S}/\text{cm}$, no road salt added). We refer to these four treatments as ambient low salt (ALS), ambient high salt (AHS), elevated low salt (ELS), and elevated high salt (EHS). In each tank, we mixed and randomly assigned more than three partial egg masses (60 tadpoles) from three different ponds to avoid parental and site effects. The frogs used in this experiment were controls that were not exposed to *Ranavirus*. Temperature was manipulated using aquarium heaters and automated controllers that adjusted temperature every 15 min; mesocosms with elevated temperature followed the same natural daily fluctuations as ambient tanks. Salinity was manipulated by adding road salt obtained from University of Connecticut facilities. The purchasing documentation indicates that the road salt contained magnesium chloride in addition to sodium chloride. We measured specific conductivity by using a YSI Pro2030 meter (YSI Inc.). A larva was removed from the tank once it reached Gosner stage 42 and then kept in plastic containers hydrated with water corresponding to the salinity treatment (high salt or low salt) until its tail had been completely absorbed. Juveniles were then temporarily housed in 5-gal (19-L) aquarium tanks corresponding to the treatment

until the experiments could take place. During this period, juvenile frogs were fed fruits flies and small crickets ad libitum. We hydrated temporary tanks with water corresponding with the salinity treatment as larvae. All frogs used in this experiment were at least 2 wk old postmetamorphosis.

Choice of Soil Experiment.—Before the experiment, we measured and recorded the mass and snout–vent length (SVL) for each frog. We placed juvenile frogs individually into standard 5-gal aquariums (41 × 21 × 26 cm) containing sand treated with low salinity (52–102 μS/cm) or high salinity (1,900–2,000 μS/cm) water separated by a soil-level divider. We used Imagitarium White Aquarium Sand, so that the white substrate would contrast with the darker colored frogs and stand out in pictures. To treat the sand, we mixed 0.59 liters of sand and 0.24 liters of high-salt or low-salt water in a 5-gal bucket. We then poured the treated sand evenly into the side of the tank corresponding to its salinity treatment. We constructed lids of saran wrap held with rubber bands because of its transparency and ability to prevent moisture from escaping the tank. We made dividers that were 1.8 cm deep out of corrugated plastic. We used Bushnell HD trophy cameras to monitor the frogs' movements, set on a time lapse at 1-min intervals for a period of 4 h. We left the room after tanks and cameras were set up, and no one was allowed into the room until the 4-h period was over. For each 4-h period, we ran 12 tanks at a time with one camera monitoring each tank and each tank containing one frog. We labeled the tanks 1–12, with each frog being randomly assigned a tank number. We tied Bushnell cameras to polyvinyl chloride poles located 140 cm above each tank and positioned the cameras facing downward. The room was illuminated with white light by using two clamp lights pointed upward toward the center of the ceiling, to mitigate any directional bias from our light source. Air temperature of the room was maintained between 21.2 and 23.1°C. The cardinal direction each tank was facing also was randomized. We conducted the experiment over the course of 3 days by using 21 animals per treatment, for a total sample size of 84 animals.

Desiccation Experiment.—We constructed 24 wire mesh desiccation chambers (5 cm × 5 cm × 5 cm), with each chamber suspended by a wire hook fashioned from a paperclip. Before the experiment, juvenile frogs were hydrated for 12 h in individual containers containing water corresponding to the salinity treatment (high salt or low salt) in which they were raised. We measured and recorded the initial mass and SVL for each individual frog and then randomly placed it into a desiccation chamber. We then recorded the mass of each frog at 20-min intervals until 20% of body mass was lost. Once a frog reached this threshold, it was immediately rehydrated. We conducted four trials over the course of 2 days, with a total sample size of 93 animals ($N_{ALS} = 24$, $N_{AHS} = 24$, $N_{EHS} = 22$, $N_{ELS} = 23$). The soil choice experiment took place first, with all individual frogs that took part in the first experiment also taking part in the evapotranspiration experiment. The two experiments took place several days apart, and we know of no effect of the soil choice experiment that could have affected the results of the evapotranspiration experiment. All frogs were euthanized after the conclusion of the experiments.

Data Analysis.—Photo processing included assigning each photo a binary code, with 1 being the frog was located on high salinity soil and 0 being the frog was located on low salinity soil. We deemed a photo usable for data analysis if the side the frog was sitting on could be clearly identified in the picture. Unusable photos were often the result of overexposure or frogs clinging to the tank glass rather than sitting on either of the soil treatments.

Frogs with <130 usable photos were excluded from data analysis. We also did not analyze the first 30 min of each 4-h period, to give frogs time to acclimate to the tank environment. The remaining 210 min was split into seven time intervals that were 30 min in length. We ran a general linear model with a quasibinomial link function to account for the proportional response, which was the proportion of minutes a frog spent on soil treated with high salt water. Proportions were calculated by taking the average value of the binary code assigned to each photo. Omitted photos were not included in the averages; thus, not all means are out of 30 observations. We tested how this choice of high salt soil differed based on the aquatic treatments of salt and temperature and whether the response changed over time. We extracted an analysis of variance (ANOVA) table from this model and plotted estimated marginal means in the figures.

We calculated desiccation rate for each frog using the following equation:

$$\frac{W_0 - W_1}{[SA \times (T_1 - T_0)]}$$

where W_0 and W_1 represent initial and final mass (g), respectively; T_0 and T_1 represent initial and final time (min), respectively; and SA represents surface area (Rudin-Bitterli et al., 2020). Surface area for each frog was estimated as $1.117 \times W_0^{0.683}$, where W_0 represents the initial mass (g; Hutchison et al., 1968). We used a two-way ANOVA extracted from a general linear model to test the effects of aquatic treatments on desiccation rate. The response was log transformed to meet assumptions of normality and equal variance. We also used a two-way ANOVA extracted from a general linear model to test for significant differences in initial mass between aquatic treatments. We also ran a separate two-way ANOVA to test for significant differences in SVL between aquatic treatments. Statistical analyses were performed using RStudio version 4.0.3 (R Studio Team, 2020).

RESULTS

Choice of Soil Experiment.—In total, 13,738 photos were classified as useable for data analysis and 69 of 84 frogs had >130 usable photos ($N_{ALS} = 14$, $N_{AHS} = 16$, $N_{EHS} = 19$, $N_{ELS} = 20$). We found that salinity treatment as larvae significantly affected choice of soil as juveniles ($P = 0.0004$; Table 1). Tadpoles raised in high salt conditions spent an average of 56.86% of the time on the high salt side of the tank. Tadpoles raised in low salt conditions spent an average of 57.28% of the time on the low salt side of the tank. Temperature treatment as larvae did not affect choice of soil as juveniles ($P = 0.5658$; Table 1). Mass was also not a significant factor ($P = 0.2867$). There was also no significant difference between 30-min time intervals ($P = 0.9946$; Table 1). The temperature × salinity interaction was statistically significant ($P = 0.0046$; Table 1; Fig. 1). We found no statistical significance in the temperature × time interval interaction ($P = 0.6019$; Table 1), salt × time interval interaction ($P = 0.6779$; Table 1), or temperature × salt × time interval interaction ($P = 0.9864$; Table 1).

Desiccation Experiment.—We found that salinity treatment as larvae did not affect desiccation rate as juvenile frogs ($F_{1,89} = 0.112$, $P = 0.738$; Table 2). Temperature treatment as larvae also did not affect desiccation rate as juveniles ($F_{1,89} = 0.250$, $P = 0.619$; Table 2). Temperature × salinity interaction was not statistically significant ($F_{1,89} = 0.706$, $P = 0.403$; Table 2; Fig. 2). Frogs used in this experiment were not uniform in mass and

TABLE 1. Analysis of deviance table for the behavioral choice of soil experiment that tests the proportion of minutes that juvenile Wood Frogs spent on soil treated with high salt water as a result of temperature (temp) and salt treatments experienced as tadpoles and over 30-min time intervals.

	df	Deviance	Residual df	Residual deviance	Chi square <i>P</i> value
Null			482	442.3	
Temp	1	0.2355	481	442.0	0.5658
Salt	1	9.0197	480	433.0	0.0004
Time interval	6	0.4965	474	432.5	0.9946
Temp : salt	1	5.7454	473	426.8	0.0046
Temp : time interval	6	3.2532	467	423.5	0.6019
Salt : time interval	6	2.8394	461	420.7	0.6799
Temp : salt : time interval	6	0.6989	455	420.0	0.9864

length at the time of the experiment, thus accounting for these size differences is important. Individuals raised in high salt had greater initial mass ($F_{1,89} = 9.358$; $P = 0.003$) and SVL ($F_{1,89} = 11.524$; $P = 0.001$) than individuals raised in low salt. Individuals raised in elevated temperature had greater initial mass ($F_{1,89} = 20.733$; $P < 0.001$) and SVL ($F_{1,89} = 10.100$; $P = 0.002$) than individuals raised in ambient temperature conditions.

DISCUSSION

As salinization of natural systems extends from aquatic systems into terrestrial systems, amphibians that live in both systems are a focal group for understanding ecological consequences of salinization. Most importantly, our experiment demonstrates that Wood Frogs can distinguish between soils that differ in salinity. Although salinization of terrestrial environments is a novel condition, the ability of frogs to detect this change in the environment is a key piece of information for understanding responses of individuals and populations to these changing environmental conditions. Furthermore, our behavioral choice experiment indicates that salinity levels experienced as larvae changes behavioral responses of juveniles when selecting terrestrial soils. We found that aquatic salinity levels correlated with the choice of soil according to our predictions, with Wood Frogs raised in high salt conditions spending the majority of time on high salinity soils and frogs raised in low salt conditions spending the majority of time on low salinity soils. As expected, we did not detect a significant relationship between temperature experienced as a larva and

choice of saline soil as a juvenile. However, we did detect a significant interaction between temperature and salinity, contrary to our hypothesis. Although larval treatment had a significant effect on individual mass, this was not a significant factor on juvenile choice of soil. We conclude that juvenile Wood Frogs can detect and avoid salts in terrestrial soils and that this avoidance behavior differs depending on salinity conditions in which they were raised in as larvae.

Previous studies have demonstrated that epithelial Na^+ transport acts in a chemosensory function, allowing amphibians to “taste” Na^+ in hydration sources. (Hoff and Hillyard, 1993; Nagai et al., 1999). Our research does not address the mechanism by which individuals detect differences in salinity among soils, but chemosensory functions are likely a predominant cue for distinguishing features of terrestrial habitats. We may speculate that juvenile Wood Frogs could be acclimated to certain ionic conditions based on the treatment in which they were raised as larvae that, in turn, influences juvenile choice of soil. Previous studies have suggested that larval amphibians in coastal environments have an increased abundance of aquaporins and ion pumps (e.g., $\text{Na}^+\text{-K}^+$ ATPase) in the gills, which improves regulation of internal water and ion concentration (Bernabò et al., 2013; Wu et al., 2014). In Green Tree Frogs (*Hyla cinerea*), differences in female oviposition site selection have been detected between coastal and inland populations across a salinity gradient (Albecker and McCoy, 2017). This difference would be unexpected if salt was equally lethal to eggs and offspring of both populations. This indicates a greater salt tolerance in coastal populations and that this tolerance influences oviposition site selection (Albecker and McCoy, 2017).

An alternative mechanism for our behavioral results may be due to differences in internal Na^+ concentrations between treatments. Terrestrial amphibians such as Red-Spotted Toads (*Anaxyrus punctatus*) have been previously shown to avoid solutions that are hypertonic to their body fluids to avoid osmotic dehydration (Brekke et al., 1991). We also speculate that larvae raised in high salt conditions may possess elevated internal concentrations of Na^+ that persist into postmetamorphic stages. These larvae may have a greater resistance to

TABLE 2. ANOVA table for the desiccation experiment that compares juvenile desiccation rate as a result of temperature (temp) and salt treatments experienced as tadpoles.

	df	Sum square	Mean square	<i>F</i> value	<i>P</i> value
Temp	1	0.002	0.002	0.250	0.619
Salt	1	0.001	0.001	0.112	0.738
Temp:salt	1	0.005	0.005	0.706	0.403
Residuals	89	0.589	0.007		

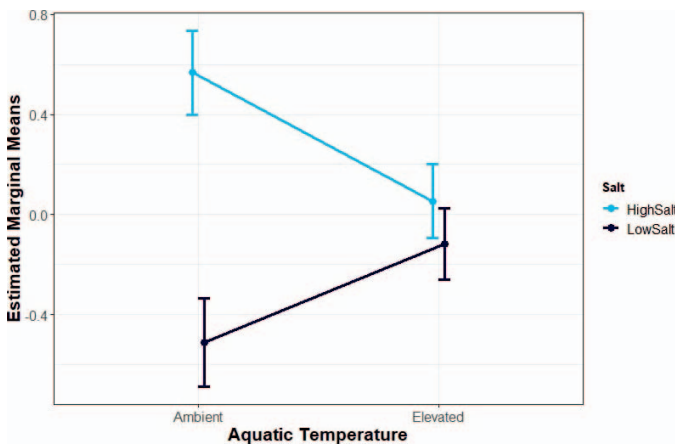


FIG. 1. Transformed estimated marginal means of the proportion of minutes juvenile Wood Frogs spent on high salinity soils vs. salinity treatment as larvae (salt) for elevated and ambient temperature treatments (aquatic temperature). Temperature \times salinity interaction was significant. Error bars represent \pm SE.

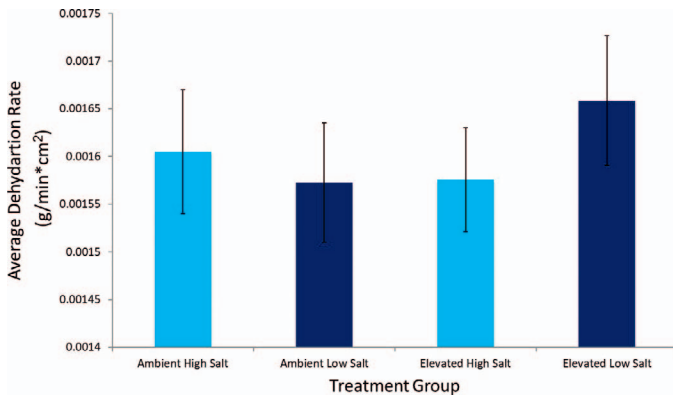


FIG. 2. Mean dehydration rate of AHS, ALS, EHS, and ELS treatment groups. No significant difference in dehydration rate was found among the four groups. Error bars represent \pm SE.

osmotic dehydration as juveniles and therefore have a greater tolerance for higher salinity terrestrial environments.

Another possible explanation is that our results are related to differences in hormone levels between salinity treatments. The mineralocorticoid aldosterone is the primary hormone responsible for maintaining salt and water balance in amphibians and all terrestrial vertebrates, although other hormones such as vasopressin also play a role (Garty and Palmer, 1997). Aldosterone stimulates transepithelial Na^+ transport by increasing apical membrane permeability through the activation of ENaCs (Garty, 2000). Frogs raised in high salt conditions may have a reduced baseline aldosterone level and thus a reduced ability to absorb Na^+ , at least in the short term. An increase in Na^+ absorption takes several hours to develop: first, with an “early response” of 1.5–3 h wherein preexisting Na^+ transport channels are activated and second, with a “late response” of 6–24 h wherein the induction of $\text{Na}^+\text{-K}^+$ ATPase pumps and ENaC subunits occurs (Garty and Palmer, 1997). Although our results did not indicate significant shifts in juvenile choice of soil over time, additional research is needed to determine whether such changes occur over time periods longer than what we tested here because of hormonal responses to a novel test environment. The proximate cause of our behavioral results also may be due to a multitude of factors arising from an adaptive response to larval salinity conditions.

Cl^- concentration also may play a role in detection and avoidance behavior. Previous research has found that exposure to deionized water resulted in increased MR cell recruitment (Budtz et al., 1995). A low recruitment of MR cells due to high salinity conditions may result in a reduced ability to regulate Cl^- . Additional research describing how salinity conditions influence the development of epithelial Na^+ channels during metamorphosis would be informative for understanding the mechanism for how frogs detect salinity in terrestrial environments. It is not yet known whether early-life exposure to elevated salinity has an effect on the phenotypic plasticity of individuals later in life, although the possibility has been suggested (Hopkins et al., 2016). Alternatively, our results may have been influenced by more immediate factors rather than larval conditions, namely the soils frogs were housed in before the experiment. In a natural wetland, ponds artificially salinized by road salt will likely be surrounded by salinized soils (Lundmark and Olafsson, 2007; Findlay and Kelly, 2011). Therefore, we choose to maintain the salinity treatment postmetamorphosis to simulate terrestrial conditions experi-

enced by juvenile Wood Frogs in a salinized natural wetland. This decision is important because of the many physiological changes to chemosensitivity and ion transport undergone during metamorphosis (Alvarado and Moody, 1970).

It is worth noting the behavioral response appears muted in Wood Frogs raised in elevated temperature treatments (Fig. 1). Chemosensitivity is known to be influenced by temperature (Santin et al., 2013). Experimental tests by Green et al. (2019) suggest that at higher temperatures, larval Green Frogs (*Lithobates clamitans*) experience greater mortality because of increased Cl^- concentrations than at lower temperatures, suggesting a potentiation of Cl^- toxicity with increasing temperature. Whether this effect carries over to later developmental stages is not known, but if true, juvenile frogs also may have an increased sensitivity to Cl^- and an increased ability to detect and avoid salt in terrestrial soils. However, the exact physiological mechanism behind this is not known because the long-term effects of larval temperature conditions on ion transport and chemosensory function have not been well studied.

Contrary to our hypothesis, the results of our desiccation experiment suggest that conditions experienced as larvae have no significant effect on desiccation rate as juveniles. We did find that in both temperature treatments, larvae raised in high salt conditions had significantly higher initial mass and SVL than larvae raised in low salt conditions. These results are backed by previous studies that found that as conductivity increased, larval Wood Frog size also increased (Dananay et al., 2015). We also found that in both salinity treatments, larvae raised in elevated temperatures had significantly higher initial mass and SVL than larvae raised in ambient temperatures. This result was likely due to the well-established fact that larval amphibians raised at colder temperatures have longer developmental periods than those raised at warmer temperatures (Smith-Gill and Berven, 1979; Álvarez and Nicieza, 2002).

Such anthropogenically induced high salinity soil conditions are a new phenomenon in the northeastern United States (Jackson and Jobbágy, 2005; Kaushal et al., 2005). As road salt applications increase, salinity increases not only in wetlands but also in terrestrial soils surrounding wetlands (Lundmark and Olafsson, 2007; Green et al., 2008; Findlay and Kelly, 2011). Our results suggest detection and avoidance capabilities are already in place in juvenile Wood Frogs, although we did not detect differences in the frogs’ ability to physiologically prevent water loss. These behavioral differences in choice of soil in juveniles may have other potential behavioral and ecological consequences, such as influencing patterns of dispersal postmetamorphosis. Our results suggest that larval Wood Frogs in high salinity roadside ponds may choose to remain on high salinity soils as juveniles, and these high salinity soils are likely to be close to the road and to the wetland from which the juveniles emerged. A shift in patterns of dispersal may lead to other potential carryover effects such as increased risk of predation (Rittenhouse et al., 2009), increased road mortality in juveniles, or reduced gene flow if juveniles are less likely to disperse from a natal wetland. Conservation strategies for amphibians are likely to be enhanced by integrating behavior and physiology (Walls and Gabor, 2019). Our findings presented here suggest that the conservation of amphibians within environments experiencing salinization will benefit from additional research that integrates behavior and physiology.

Acknowledgments.—We thank A. Benedetti, M. O'Connor, M. Cisse, M. Castonguay, and M. Parsley for assistance raising tadpoles in aquatic mesocosm portion of the study. Funding provided by National Science Foundation Research Experiences for Undergraduates program and National Science Foundation award 1754474. Research completed under animal care protocol A20-017.

LITERATURE CITED

- ADOLPH, E. F. 1932. The vapor tension relations of frogs. *Biological Bulletin* 62:112–125.
- ALBECKER, M. A., AND M. W. MCCOY. 2017. Adaptive responses to salinity stress across multiple life stages in anuran amphibians. *Frontiers in Zoology* 14:40.
- ALVARADO, R., AND A. MOODY. 1970. Sodium and chloride transport in tadpoles of the bullfrog *Rana catesbeiana*. *American Journal of Physiology-Legacy Content* 218:1510–1516.
- ÁLVAREZ, D., AND A. G. NICIEZA. 2002. Effects of temperature and food quality on anuran larval growth and metamorphosis. *Functional Ecology* 16:640–648.
- ARDÓN, M., A. M. HELTON, AND E. S. BERNHARDT. 2018. Salinity effects on greenhouse gas emissions from wetland soils are contingent upon hydrologic setting: a microcosm experiment. *Biogeochemistry* 140: 217–232.
- BALDWIN, R. F., AND P. G. DEMAYNADIER. 2009. Assessing threats to pool-breeding amphibian habitat in an urbanizing landscape. *Biological Conservation* 142:1628–1638.
- BASTVIKEN, D., P. SANDÉN, T. SVENSSON, C. STAHLBERG, M. MAGOUNAKIS, AND G. ÖBERG. 2006. Chloride retention and release in a boreal forest soil: effects of soil water residence time and nitrogen and chloride loads. *Environmental Science and Technology* 40:2977–2982.
- BELLAKHAL, M., A. NEVEU, M. FARTOUNA-BELLAKHAL, H. MISSAOUI, AND L. ALEVA. 2014. Effects of temperature, density and food quality on larval growth and metamorphosis in the north African green frog *Pelophylax saharicus*. *Journal of Thermal Biology* 45:81–86.
- BERNABO, I., A. BONACCI, F. COSCARELLI, M. TRIPEPI, AND E. BRUNELLI. 2013. Effects of salinity stress on *Bufo balearicus* and *Bufo* tadpoles: tolerance, morphological gill alterations and Na^+/K^+ -ATPase localization. *Aquatic Toxicology* 132:119–133.
- BRADFORD, D. F. 1984. Water and osmotic balance in overwintering tadpoles and frogs, *Rana muscosa*. *Physiological Zoology* 57:474–480.
- BREKKE, D. R., S. D. HILLYARD, AND R. M. WINOKUR. 1991. Behavior associated with the water absorption response by the toad, *Bufo punctatus*. *Copeia* 1991:393–401.
- BROWN, S. C., E. A. HORGAN, L. M. SAVAGE, AND P. S. BROWN. 1986. Changes in body water and plasma constituents during bullfrog development: effects of temperature and hormones. *Journal of Experimental Zoology* 237:25–33.
- BUDTZ, P. E., B. C. CHRISTOFFERSEN, J. S. JOHANSEN, I. SPIES, AND N. J. WILLUMSEN. 1995. Tissue kinetics, ion transport, and recruitment of mitochondria-rich cells in the skin of the toad (*Bufo bufo*) in response to exposure to distilled water. *Cell and Tissue Research* 280:65–75.
- DALEY, M. L., J. D. POTTER, AND W. H. McDOWELL. 2009. Salinization of urbanizing New Hampshire streams and groundwater: effects of road salt and hydrologic variability. *Journal of the North American Benthological Society* 28:929–940.
- DANANAY, K. L., K. L. KRYNAK, T. J. KRYNAK, AND M. F. BENARD. 2015. Legacy of road salt: apparent positive larval effects counteracted by negative postmetamorphic effects in wood frogs. *Environmental Toxicology and Chemistry* 34:2417–2424.
- DENOËL, M., M. BICHOT, G. F. FICETOLA, J. DELCOURT, M. YLIEFF, P. KESTEMONT, AND P. PONCIN. 2010. Cumulative effects of road de-icing salt on amphibian behavior. *Aquatic Toxicology* 99:275–280.
- DENVER, R. J. 1997. Proximate mechanisms of phenotypic plasticity in amphibian metamorphosis. *American Zoologist* 37:172–184.
- DUARTE, H., M. TEJEDO, M. KATZENBERGER, F. MARANGONI, D. BALDO, J. F. BELTRÁN, D. A. MARTÍ, A. RICHTER-BOIX, AND A. GONZALEZ-VOYER. 2012. Can amphibians take the heat? Vulnerability to climate warming in subtropical and temperate larval amphibian communities. *Global Change Biology* 18:412–421.
- FINDLAY, S. E. G., AND V. R. KELLY. 2011. Emerging indirect and long-term road salt effects on ecosystems. *Annals of the New York Academy of Sciences* 1223:58–68.
- FORMAN, R. T. T., AND L. E. ALEXANDER. 1998. Roads and their major ecological effects. *Annual Review of Ecology and Systematics* 29: 207–231.
- GARTY, H. 2000. Regulation of the epithelial Na^+ channel by aldosterone: open questions and emerging answers. *Kidney International* 57: 1270–1276.
- GARTY, H., AND L. G. PALMER. 1997. Epithelial sodium channels: function, structure, and regulation. *Physiological Reviews* 77:359–396.
- GRANT, E. H. C., D. A. W. MILLER, B. R. SCHMIDT, M. J. ADAMS, S. M. AMBURGEY, T. CHAMBERT, S. S. CRUICKSHANK, R. N. FISHER, D. M. GREEN, B. R. HOSSACK, ET AL. 2016. Quantitative evidence for the effects of multiple drivers on continental-scale amphibian declines. *Scientific Reports* 6:25625.
- GREEN, F. B., A. G. EAST, AND C. J. SALICE. 2019. Will temperature increases associated with climate change potentiate toxicity of environmentally relevant concentrations of chloride on larval green frogs (*Lithobates clamitans*)? *Science of the Total Environment* 682: 282–290.
- GREEN, S. M., R. MACHIN, AND M. S. CRESSER. 2008. Effect of long-term changes in soil chemistry induced by road salt applications on N-transformations in roadside soils. *Environmental Pollution* 152:20–31.
- HALL, E. M., S. P. BRADY, N. M. MATTHEUS, R. L. EARLEY, M. DIAMOND, AND E. J. CRESPI. 2017. Physiological consequences of exposure to salinized roadside ponds on wood frog larvae and adults. *Biological Conservation* 209:98–106.
- HALL, E. M., J. L. BRUNNER, B. HUTZENBILER, AND E. J. CRESPI. 2020. Salinity stress increases the severity of ranavirus epidemics in amphibian populations. *Proceedings of the Royal Society B* 287:20200062.
- HILLYARD, S. D. 1999. Behavioral, molecular and integrative mechanisms of amphibian osmoregulation. *Journal of Experimental Zoology* 283: 662–674.
- HINTZ, W. D., AND R. A. RELYEA. 2019. A review of the species, community, and ecosystem impacts of road salt salinisation in fresh waters. *Freshwater Biology* 64:1081–1097.
- HOFF, K., AND S. D. HILLYARD. 1993. Toads taste sodium with their skin: sensory function in a transporting epithelium. *Journal of Experimental Biology* 183:347–351.
- HOPKINS, G. R., E. D. BRODIE, JR., L. A. NEUMAN-LEE, S. MOHAMMADI, G. A. BRUSCH IV, Z. M. HOPKINS, AND S. S. FRENCH. 2016. Physiological responses to salinity vary with proximity to the ocean in a coastal amphibian. *Physiological and Biochemical Zoology* 89:322–330.
- HULING, E. E., AND T. C. HOLLOCHER. 1972. Groundwater contamination by road salt: steady-state concentrations in east central Massachusetts. *Science* 176:288–290.
- HUTCHISON, V. H., W. G. WHITFORD, AND M. KOHL. 1968. Relation of body size and surface area to gas exchange in anurans. *Physiological Zoology* 41:65–85.
- JACKSON, R. B., AND E. G. JOBBÁGY. 2005. From icy roads to salty streams. *Proceedings of the National Academy of Sciences of the United States of America* 102:14487–14488.
- JARED, S. R., AND J. P. RAO. 2017. Transepithelial sodium transport across frog skin. *Advances in Physiology Education* 41:444–447.
- JØRGENSEN, C. B. 1991. Water and salt balance at low temperature in a cold temperate zone anuran, the toad *Bufo*. *Comparative Biochemistry and Physiology Part A: Physiology* 100:377–384.
- KARRAKER, N. E., J. P. GIBBS, AND J. R. VONESH. 2008. Impacts of road deicing salt on the demography of vernal pool-breeding amphibians. *Ecological Applications* 18:724–734.
- KAUSHAL, S. S., P. M. GROFFMAN, G. E. LIKENS, K. T. BELT, W. P. STACK, V. R. KELLY, L. E. BAND, AND G. T. FISHER. 2005. Increased salinization of fresh water in the northeastern United States. *Proceedings of the National Academy of Sciences of the United States of America* 102: 13517–13520.
- KELLY, V. R., G. M. LOVETT, K. C. WEATHERS, S. E. FINDLAY, D. L. STRAYER, D. J. BURNS, AND G. E. LIKENS. 2008. Long-term sodium chloride retention in a rural watershed: legacy effects of road salt on streamwater concentration. *Environmental Science and Technology* 42:410–415.
- KÖHLER, A., J. SADOWSKA, J. OLSZEWSKA, P. TRZECIAK, O. BERGER-TAL, AND C. R. TRACY. 2011. Staying warm or moist? Operative temperature and thermal preferences of common frogs (*Rana temporaria*), and effects on locomotion. *Herpetological Journal* 21:17–26.
- LUNDMARK, A., AND B. OLOFSSON. 2007. Chloride deposition and distribution in soils along a deiced highway – assessment using different methods of measurement. *Water, Air, and Soil Pollution* 182:173–185.

- MILOTIC, D., M. MILOTIC, AND J. KOPRIVNIKAR. 2017. Effects of road salt on larval amphibian susceptibility to parasitism through behavior and immunocompetence. *Aquatic Toxicology* 189:42–49.
- NAGAI, T., H. KOYAMA, K. V. S. HOFF, AND S. D. HILLYARD. 1999. Desert toads discriminate salt taste with chemosensory function of the ventral skin. *Journal of Comparative Neurology* 408:125–136.
- PATRICK, D. A., E. B. HARPER, M. L. HUNTER, JR., AND A. J. CALHOUN. 2008. Terrestrial habitat selection and strong density-dependent mortality in recently metamorphosed amphibians. *Ecology* 89:2563–2574.
- R STUDIO TEAM. 2020. RStudio: integrated development for R. R Studio, Inc., Boston, USA.
- RITTENHOUSE, T. A. G., AND R. D. SEMLITSCH. 2009. Behavioral response of migrating wood frogs to experimental timber harvest surrounding wetlands. *Canadian Journal of Zoology* 87:618–625.
- RITTENHOUSE, T. A. G., M. C. DOYLE, C. R. MANK, B. B. ROTHERMEL, AND R. D. SEMLITSCH. 2004. Substrate cues influence habitat selection by spotted salamanders. *Journal of Wildlife Management* 68:1151–1158.
- RITTENHOUSE, T. A. G., E. B. HARPER, L. R. REHARD, AND R. D. SEMLITSCH. 2008. The role of microhabitats in the desiccation and survival of anurans in recently harvested oak–hickory forest. *Copeia* 2008:807–814.
- RITTENHOUSE, T. A. G., R. D. SEMLITSCH, AND F. R. THOMPSON III. 2009. Survival cost associated with wood frog breeding migrations: effects of timber harvest and drought. *Ecology* 90:1620–1630.
- ROBINSON, H. K., E. A. HASENMUELLER, AND L. G. CHAMBERS. 2017. Soil as a reservoir for road salt retention leading to its gradual release to groundwater. *Applied Geochemistry* 83:72–85.
- RUDIN-BITTERLI, T. S., J. P. EVANS, AND N. J. MITCHELL. 2020. Geographic variation in adult and embryonic desiccation tolerance in a terrestrial-breeding frog. *Evolution* 74:1186–1199.
- RUTHSATZ, K., M. A. PECK, K. H. DAUSMANN, N. M. SABATINO, AND J. GLOS. 2018. Patterns of temperature induced developmental plasticity in anuran larvae. *Journal of Thermal Biology* 74:123–132.
- SANTIN, J. M., K. C. WATTERS, R. W. PUTNAM, AND L. K. HARTZLER. 2013. Temperature influences neuronal activity and CO₂/pH sensitivity of locus coeruleus neurons in the bullfrog, *Lithobates catesbeianus*. *American Journal of Physiology: Regulatory, Integrative and Comparative Physiology* 305:R1451–R1464.
- SMITH-GILL, S. J., AND K. A. BERVEN. 1979. Predicting amphibian metamorphosis. *American Naturalist* 113:563–585.
- SPOTILA, J. R., AND E. N. BERMAN. 1976. Determination of skin resistance and the role of the skin in controlling water loss in amphibians and reptiles. *Comparative Biochemistry and Physiology Part A: Physiology* 55:407–411.
- STUART, S. N., J. S. CHANSON, N. A. COX, B. E. YOUNG, A. S. L. RODRIGUES, D. L. FISCHMAN, AND R. W. WALLER. 2004. Status and trends of amphibian declines and extinctions worldwide. *Science* 306:1783–1786.
- TENNESSEN, J. B., S. E. PARKS, AND T. LANGKILDE. 2014. Traffic noise causes physiological stress and impairs breeding migration behaviour in frogs. *Conservation Physiology* 2:cou032.
- TOLEDO, R. C., AND C. JARED. 1993. Cutaneous adaptations to water balance in amphibians. *Comparative Biochemistry and Physiology Part A: Physiology* 105:593–608.
- TROMBULAK, S. C., AND C. A. FRISSELL. 2000. Review of ecological effects of roads on terrestrial and aquatic communities. *Conservation Biology* 14:18–30.
- WAKE, D. B., AND V. T. VREDENBURG. 2008. Are we in the midst of the sixth mass extinction? A view from the world of amphibians. *Proceedings of the National Academy of Sciences of the United States of America* 105:11466–11473.
- WALLS, S. C., AND C. R. GABOR. 2019. Integrating behavior and physiology into strategies for amphibian conservation. *Frontiers in Ecology and Evolution* 7:234.
- WU, C. S., W. K. YANG, T. H. LEE, I. GOMEZ-MESTRE, AND Y. C. KAM. 2014. Salinity acclimation enhances salinity tolerance in tadpoles living in brackish water through increased Na⁺, K⁺-ATPase expression. *Journal of Experimental Zoology Part A: Ecological Genetics and Physiology* 321:57–64.

Accepted: 11 May 2021.

Published online: 11 March 2022.