Effects of Freshwater Salinization and Biotic Stressors on Amphibian Morphology

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Organisms are commonly exposed to numerous stressors that induce behavioral, physiological, or morphological changes in some combination. At northern temperate latitudes, de-icing agents (primarily sodium chloride, NaCl) are a major stressor to species in freshwater ecosystems. Species-specific responses to road salt toxicity range from lethal to sublethal effects, but it remains unclear how these effects interact with biotic stressors. Morphology can be quite sensitive to environmental changes, yet we know little about how it is affected by road salt exposure. We exposed Wood Frog tadpoles (*Rana sylvatica*) to two road salt formulations (NaCl and a mixture of NaCl, MgCl₂, and KCl), each at three concentrations (200, 600, and 1000 mg Cl⁻/L), crossed with three biotic stressor levels (predator cue, competition, and a no-stressor control). We then measured the impacts on relative morphology (snout–vent length, body width, forelimb length, hindlimb length, hindlimb width) of the emerging metamorphs. Salt concentration and biotic stressors both impacted relative morphology, but their effects did not interact. Exposure to road salts increased relative snout–vent length (SVL) and body width. In contrast, competition induced relatively shorter SVL and forelimb length while predator cues induced relatively longer hindlimbs and narrower forelimbs. This is the first discovery that road salts can induce changes in amphibian morphology and that these effects are independent of changes induced by biotic stressors. Future research should examine the effects on overwintering success and future fitness in amphibians as well as the impacts of salt on the morphology of other aquatic taxa that are being exposed to freshwater salinization.

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Materials and Methods

Experimental Design.—To determine effects of road salt and biotic stressors on amphibian morphology, we measured external morphology of recently metamorphosed Wood Frogs preserved from Jones et al. (2017). In brief, Jones et al. (2017) conducted a factorial experiment that used seven salt treatments: 1) a no-salt control, 2) three concentrations of NaCl (95–100% pure; 200, 600, and 1000 mg Cl/L), and 3) three concentrations of a salt mixture containing chloride-based alternatives (NaCl, MgCl₂, and KCl; 200, 600, and 1000 mg Cl/L). The seven salt treatments were crossed with two biotic stressors (predator cue, competition, and a no-stressor control). The 21 treatment combinations were replicated four times for a total of 84 experimental units.

To simulate a natural wetland, we used mesocosms, outdoor experimental systems which mimic natural conditions and serve as a bridge between field studies and highly controlled laboratory experiments. Each 90 L outdoor mesocosm served as an experimental unit and was filled with 82 L of tap water, leaf litter, and a homogenized aliquot of pond water containing periphyton, phytoplankton, and zooplankton. Tap water was left for several days to allow for the chlorine to off-gas prior to the addition of leaf litter and pond water. Newly hatched American Toad larvae and newly hatched Wood Frog larvae were also added. Initial Wood Frog and American Toad masses were 147 ± 10 and 38 ± 3 mg, respectively (mean ± SE; Jones et al., 2017); both species were at Gosner developmental stage 25 (Gosner, 1960). Competitor stress was manipulated by doubling the number of Wood Frog and toad tadpoles in the respective mesocosms, such that 20 total individuals of each species (density of 24 individuals/m²) were added to competition treatments while ten individuals of each species (density of 12 individuals/m²) were added to the predator-cue and no-stressor treatments. These densities are well within the densities observed in natural wetlands (Relyea et al., unpubl. data). Furthermore, the original experiment showed significant effects of competitive environment on Wood Frog responses to increased tadpole densities (Jones et al., 2017), indicating that even if the densities chosen do not represent true high and low densities, the higher density was enough to increase competition for resources in the mesocosms. Each mesocosm contained a single predator cage; to manipulate predator stress, we added a single dragonfly larva (Anax junius) to the appropriate cages. The dragonfly larvae were fed approximately 300 mg of larval Wood Frogs three times per week. Previous studies indicated that larval Wood Frogs show antipredator behavior and morphological changes to caged dragonfly larvae that are fed this biomass of prey (Schoepfner and Relyea, 2008).

Wood Frogs were removed from the mesocosms daily as they metamorphosed, which we defined as having four legs and any amount of tail. We housed the metamorphs in the laboratory until tail sorption resulted in tail lengths of <2 mm, at which time they were euthanized and preserved in a 10% formalin solution. The experiment ended at day 49 when the last individual metamorphosed. Biotic stressors had significant effects on amphibian survival, time to metamorphosis, and mass at metamorphosis, but there was no effect of salt or salt-by-environment interactions (Jones et al., 2017). While there was a significant effect of environment on survival, 94–98% of amphibians remained alive to the end of the experiment (Jones et al., 2017). Therefore, we did not consider any effect of survival on morphology. In the current study, we examined the impacts of the 21 treatment combinations on the relative morphology of the Wood Frogs.

Morphometric Analysis.—Morphometric data for each Wood Frog were obtained using an Olympus SZX16 microscope and cellSens software. To limit exposure to formaldehyde, frogs were removed from preservation vials and placed in tap water. Frogs were then towel dried, weighed, and photo-
To evaluate the effect of road salt treatment, environment (biotic stressors), and the mass covariate, environment (biotic stressors), and the mass covariate, we performed subsequent analyses of covariance (ANCOVAs) for each of the six body measurements, while excluding all of the mass-by-treatment interactions since they were not significant in the MANCOVA. When main effects were significant, we conducted a Dunnett test to compare the effects of each salt treatment to the no-salt control and each biotic stressor to the no-stressor control. Statistical analyses were all conducted in R version 3.6.1 (R Core Team, 2019).

**RESULTS**

MANCOVA identified significant effects of road salt treatment, environment (biotic stressors), and the mass covariate, but there was no salt-by-environment interaction (Table 1). The subsequent ANCOVAs indicated that the salt treatments affected relative SVL and body width (Table 2; Fig. 2). Relative SVL increased in all concentrations of NaCl (low: $P = 0.008$; medium: $P = 0.039$; high: $P = 0.021$); it also marginally increased in the low ($P = 0.060$) and high ($P = 0.069$) concentrations of the salt mixture. Body width increased (or marginally increased) in all concentrations of NaCl (low: $P = 0.005$; medium: $P = 0.060$; and high: $P = 0.003$); it also increased when exposed to the highest concentration of the salt mixture ($P = 0.002$). In terms of actual amount of

Table 1. Results of the multivariate analysis of covariance (MANCOVA) for log-transformed morphological variables of post-metamorphic Wood Frogs. The model was analyzed using salt treatment and tadpole environment as main effects, mass as a covariate, and all of the possible interactions. Removing the mass-by-treatment interactions in the MANCOVA had no qualitative effect on the effects of salt, environment, or their interaction.

|             | df | Wilks  | $P$  |
|-------------|----|--------|------|
| Salt        | 6  | 0.194  | 0.001|
| Environment | 2  | 0.004  | <0.001|
| Mass        | 1  | 0.039  | <0.001|
| Salt $\times$ Environment | 12 | 0.174  | 0.315|
| Salt $\times$ Mass | 6  | 0.468  | 0.686|
| Environment $\times$ Mass | 2  | 0.834  | 0.846|
| Salt $\times$ Environment $\times$ Mass | 12 | 0.204  | 0.539|
| Residuals   | 42 |        |      |

Table 2. Results of the analyses of covariance (ANCOVA) for each of the six log-transformed Wood Frog metamorph body measurements (SVL, body width, forelimb length, forelimb width, hindlimb length, hindlimb width). Each ANCOVA included salt treatment and tadpole environment as main effects and log-transformed mass as a covariate. Interactions were not included in the ANCOVAs since they were not significant in the MANCOVA (Table 1).

|             | df | $F$    | $P$  |
|-------------|----|--------|------|
| Snout–vent length |    |        |      |
| Salt        | 6  | 2.379  | 0.037|
| Environment | 2  | 2.544  | 0.085|
| Mass        | 1  | 526.480| <0.001|
| Body width  |    |        |      |
| Salt        | 6  | 3.473  | 0.004|
| Environment | 2  | 0.923  | 0.402|
| Mass        | 1  | 231.514| <0.001|
| Forelimb length |    |        |      |
| Salt        | 6  | 1.542  | 0.177|
| Environment | 2  | 2.918  | 0.060|
| Mass        | 1  | 154.994| <0.001|
| Forelimb width |    |        |      |
| Salt        | 6  | 0.892  | 0.506|
| Environment | 2  | 2.861  | 0.064|
| Mass        | 1  | 192.841| <0.001|
| Hindlimb length |    |        |      |
| Salt        | 6  | 0.946  | 0.468|
| Environment | 2  | 3.731  | 0.029|
| Mass        | 1  | 217.926| <0.001|
| Hindlimb width |    |        |      |
| Salt        | 6  | 0.673  | 0.672|
| Environment | 2  | 0.512  | 0.602|
| Mass        | 1  | 211.930| <0.001|

Fig. 1. The morphological dimensions that were measured on Wood Frog metamorphs: SVL = snout–vent length, BW = body width, FL = forelimb length, FW = forelimb width, HL = hindlimb length, and HW = hindlimb width.
morphological change (using non-logged values), NaCl lengthened SVL by 1.4 to 1.7% and body width by 1.9 to 2.8%. The salt mixture lengthened SVL by 1.2 to 1.3% and body width by 2.8%. When considering jumping performance, the ratio of hindlimb to SVL decreased at most by 1.3% in both salt formulations and across concentration.

The ANCOVAs also indicated that environment marginally affected SVL while significantly affecting forelimb length, forelimb width, and hindlimb length. The induced changes we observed are likely linked to morphological changes occurring in the tadpole stage. For instance, tadpoles reared in low resources or high competition develop relatively small tails and large bodies (Relyea, 2002, 2004; Relyea and Auld, 2005; Van Buskirk, 2009). In considering the subsequent effects as metamorphs, Southern Leopard metamorphs (Rana sphenocephala) reared under competition as tadpoles exhibit relatively longer hindlimbs (Emerson, 1986). Relyea and Hoverman (2003) found that larval competition induced Treefrog (Hyla versicolor) metamorphs to develop 1% shorter relative SVL and 3% shorter relative forelimbs. These results align with our study of Wood Frogs, with metamorphs induced to have 1.6% shorter SVL and 3.1% shorter forelimbs after larval exposure to competition.

DISCUSSION

Secondary salinization of freshwater systems is an increasingly problematic global scenario (Cañedo-Argüelles et al., 2019). To understand how road salt contamination might influence species' responses to natural stressors, we explored the effects of road salt and biotic stressors on Wood Frog morphology and found that both factors induced morphological changes.

We discovered that competition induces relatively shorter SVL and forelimbs while predator cues induce relatively narrow forelimbs and longer hindlimbs. Furthermore, road salt induced relatively longer SVL and body width. However, we did not observe any salt-by-environment interactions. This is consistent with studies looking at other sublethal effects in Rainbow Trout (Hinz and Relyea, 2017), American Toads, and Wood Frogs (Jones et al., 2017), both of which found no interaction between salt and biotic stressors.

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Our results demonstrate carry-over effects across metamorphosis indicating larval conditions have the potential to shape post-metamorphic life. Carry-over effects from environmental stressors early in ontogeny to later in life are found across taxa including insects (Taylor, 1988; Anholt, 1990, 1991; De Moed et al., 1997; Block and Stoks, 2005; Koenraadt et al., 2010; Stoks and Córdoba-Aguilar, 2012), mammals (Ravelli et al., 1976), birds (Haywood and Perrins, 1992; Merila and Svensson, 1997), marine invertebrates (Woollacott et al., 1989; Pechenik et al., 1998), and fishes (Brönmark and Pettersson, 1994; Royle et al., 2006; Roussel, 2007; Chivers et al., 2008). In damselflies, for example, larval competition results in later emergence and lower mass (Anholt, 1990), both of which had negative consequences for later adult fitness (Anholt, 1991; Block and Stoks, 2005; reviewed by Stoks and Córdoba-Aguilar, 2012). Predation stress in fishes induces morphological changes that are not entirely reversible (Brönmark and Pettersson, 1994; Chivers et al., 2008). For example, Crucian Carp (Carassius carassius) increase body depth in response to Northern Pike (Esox lucius) chemical cues and do not return to the same body depth as control even after 180 days in the absence of predation stress (Brönmark and Pettersson, 1994). Across a range of taxa, early ontogeny stress may have irreversible effects in later life stages.

To our knowledge, this is the first study to report on the effects of road salts on multiple morphological traits. We noted an increase in relative SVL across road salt type and concentration, which is similar to Dananay et al.’s (2015) report of a positive correlation between absolute SVL and road salt concentration in Wood Frogs from ponds in northeastern Ohio. In contrast, Kearney et al. (2016) used seawater instead of road salt and found that exposure to high larval salt concentrations (12% seawater) decreases mass and absolute SVL in freshwater Brown Treefrog (Litoria ewingii) populations. Overall, the two morphological changes that we observed were similar for each of the two salt formulations and were generally similar across the three concentrations used. This suggests there may be some generalities in how various road salts induce metamorph morphology.

A small number of amphibian studies have examined how road salts affect life history traits. For instance, Albecker and McCoy (2019) found that American Green Treefrogs (H. cinerea) in salt-adapted populations metamorphized earlier and smaller compared to salt-naive inland populations, but they maintained a constant size across experimentally elevated salt concentrations while inland frogs metamorphized much smaller in high salt concentrations than in lower concentrations. Jones et al. (2017) reported no effect of road salt on tadpole survival, activity, time to metamorphosis, or mass at metamorphosis, but indicated a significant reduction in toad activity in high NaCl (1000 mg Cl/L) treatments. Plant studies have shown elevated salt leading to phenotypic plasticity with changes in the number of leaves, leaf size (Richards et al., 2005), root structure (Echeverría et al., 2008), and plant architecture (Suter and Widmer, 2013).

Our results highlight the lasting effects of salinization on the post-metamorphic stages of animals. Similarly, other studies indicate salt to have persistent effects. For example, Karraker and Gibbs (2011) reported reduced mass by 33% in Spotted Salamanders (Ambystoma maculatum) that were exposed to high salt concentrations (945 mg Cl/L) with mass reduction continuing even when returned to low salt concentrations. Furthermore, Lambert et al. (2017) found that elevated road salt during larval development can masculinize Wood Frog metamorphs. Salt concentrations of 867 mg Cl/L decreased the ratio of female metamorphs in their experimental population by 10%. In contrast, Matlaga et al. (2014) did not report a lag effect of salt exposure with American Bullfrogs (Lithobates catesbeianus) as embryos reared in NaCl (7, 100, 500, and 1000 mg Cl/L) were not more vulnerable to future predation events. Outside of amphibians, Atlantic Salmon (Salmo salar) eggs within the first 24 h after fertilization showed global transcriptional changes when exposed to 5000 mg Cl/L of road salt (Tollefsen et al., 2015). Although it has not been determined whether the transcriptional changes have adverse effects, they are suggested to interfere with osmoregulation, ion regulation, oxidative stress, metabolism, renal function, and development in the embryos, which would likely span across ontogeny. While the research is limited, early exposure to road salt appears to have persistent effects across life stages.

The morphological changes induced following larval exposure to predation, competition, and road salt in Wood Frog metamorphs are relatively small, so it is important to consider whether these changes have ecological consequences. For example, Brady et al. (2019) reported increased body mass and farther jumping in female Wood Frogs from populations in close proximity to roads with high salt concentrations. The morphological changes in our study ranged from 1.2–2.8% for road salt and 1.5–3.1% for biotic stressors. Previous studies report a minimum of a 10% change in the ratios of relative hindlimb length to SVL between species before jumping is significantly affected (Stokely and Berberian, 1953; Zug, 1972; Emerson, 1978, 1986). Although reporting on 18 different anuran species, it is unclear how these studies might apply to the jumping ability of Wood Frogs, and, to our knowledge, there have been no jumping performance studies in Wood Frogs. Certainly, much more work needs to be done on this question, including whether sublethal salt exposures affect other traits.

**Conclusion.**—Our study discovered significant effects of biotic stressors and road salts during the larval period on the morphology of emerging metamorphs, indicating the importance of studies spanning across life stages. Future studies should examine whether similar morphological changes are also inducible in other species of amphibians and whether other aquatic species experience altered morphology from salt exposures. If so, then it is important to know what concentrations and salt formulations (many of which may contain chemical additives such as anti-caking agents) have an effect, the underlying mechanisms that cause these induced changes, and the short- and long-term impacts of these morphological changes on the performance of the induced individuals.

**DATA ACCESSIBILITY**

All data are available from the Dryad Digital Repository: https://doi.org/10.5061/dryad.8ght76kx. Unless otherwise indicated in the figure caption, the published images and illustrations in this article are licensed by the American Society of Ichthyologists and Herpetologists for use if the use includes a citation to the original source in accordance with the Creative Commons Attribution CC BY License.
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