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The Combined Effects of Road Salt and Biotic Stressors on Amphibian Sex Ratios

Sagan Leggett,[a,]* Jonathan Borrelli,[b] Devin K. Jones,[b] and Rick Relyea[a]

[a]Department of Biological Sciences, Darrin Fresh Water Institute, Rensselaer Polytechnic Institute, New York, USA
[b]Department of Biological Sciences, University of Notre Dame, Notre Dame, Indiana, USA

Abstract: Aquatic systems worldwide are threatened by the anthropogenic use of synthetic chemicals, including pesticides, pharmaceuticals, and road de-icers. Exposure to contaminants can alter the behavior, morphology, and physiology of organisms if it occurs during sensitive life stages. For instance, past studies have documented feminization of male amphibians following herbicide exposure and skewed sex ratios among amphibian populations exposed to road salt. However, many of these studies lack the complexities found within natural environments, such as competition with conspecifics or threat of predation, which are also known to influence development. Thus, it is important to understand how anthropogenic and natural stressors interact to alter animal sex ratios. Given the growing concern of secondary salinization of freshwater systems, we exposed larval wood frogs (*Rana sylvatica*) to either road salt (sodium chloride [NaCl]) or an alternative salt mixture (NaCl, magnesium chloride [$MgCl_2$], and potassium chloride [KCl]) at 3 concentrations (200, 600, and 1000 mg Cl^-/L) crossed with 3 biotic stressors (no-stressor control, competition, or predator cues) to examine their potentially interactive effects on sex. Exposure to biotic stressors and NaCl did not influence wood frog sex ratios. In contrast, tadpole exposure to the intermediate salt mixture concentration significantly reduced the proportion of female frogs. Future studies are needed to determine whether such changes in sex are widespread among sensitive species with complex life cycles, and to assess the consequences of sex ratio changes on long-term population dynamics. *Environ Toxicol Chem* 2021;40:231–235. © 2020 SETAC

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INTRODUCTION

Organisms are regularly exposed to numerous stressors, some of which are anthropogenic (Sih et al. 2004). Anthropogenic stressors continue to increase over time, and among them are global climate change, excess nutrients, invasive species, and contaminants (Marcogliese 2008; Noyes et al. 2009; Blakeslee et al. 2010; Núñez-Vazquez et al. 2011). A contaminant of growing environmental concern is road salt, the leading cause of freshwater salinization (Pulido 2016). Salts from mining, irrigation, and winter road applications make their way into streams, rivers, wetlands, and lakes, resulting in higher concentrations. For winter roads, road salt is used as a de-icer due to its ability to lower the freezing point of water on the roads (Kelly et al. 2010). The use of road salt as a de-icer was first introduced into the United States in 1938 and has dramatically increased over the decades. The United States has more than doubled its use of road salt since 1990, now applying more than 20 million metric tons annually (Collins and Russell 2009). Typically freshwater ecosystems contain approximately 20 mg Cl^-/L, but along with the increase in road salt use, this number now can reach up to 13 500 mg Cl^-/L depending on its proximity to stockpile salt pads (Hintz and Relyea 2019). Although freshwater chloride levels can increase from sewage and water softeners, modeling has shown that more than 90% of chloride levels can be attributed to the addition of road salt (Kelly et al. 2008). Similar studies have shown a direct correlation between influxes of road salt use and high chloride levels in freshwater systems throughout the world (Kelly et al. 2008; Dugan et al. 2017). The primary road salt used in the world is sodium chloride (NaCl), but the use of calcium chloride ($CaCl_2$) and magnesium chloride ($MgCl_2$) is becoming more common.

Although road salt has been applied for more than 9 decades, its effects on freshwater ecosystems have not received much research attention until the past decade (Hintz and Relyea 2019). The focus of such studies has been the lethal effects of road salts on a wide variety of aquatic taxa. However,

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* Address correspondence to legges2@rpi.edu

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there are likely important sublethal effects on aquatic organisms as well. For example, a recent study found that wood frog tadpoles exposed to 867 mg Cl^-/L of NaCl caused a 10% increase in the percentage of male wood frogs at metamorphosis compared with a low-salt control (Lambert et al. 2016). Given that the survival rates for that study were constant between the control and salt treatments, the masculinization of the frog population was not due to the differential survival of the males and females. Instead, that study suggests that the larval exposure to the salt caused a greater proportion of male wood frogs than expected. This shift to favoring males has dangerous implications for the stability of wood frog population dynamics. Fewer females in a population results in fewer offspring, which has axiomatic consequences on the survival of the species affected by this exposure. After only a few generations of having an insufficient number of males, the wood frog population could decline, thus highlighting the importance that sex ratios within species be maintained throughout time. Given this discovery, it becomes imperative that we understand how a range of NaCl concentrations affects animal sex ratios and whether altered sex ratios may also occur with other salt mixtures.

Unfortunately, the influx of salt is not the only stressor these amphibians must face; they have to endure natural stressors as well. Natural stressors include variable temperatures, competition, predation, and parasitism (Holmstrup et al. 2010), and these stressors can affect a variety of traits, such as morphology development, survival, and immune function (Relyea and Hoverman 2003; Reeve et al. 2013; Walls and Gabor 2019). Competition and predation, specifically, are very common in amphibian breeding habitats and have also been shown to interact with anthropogenic stressors, such as pesticides, to make them more or less lethal to amphibians (Relyea 2018). To understand fully how amphibians are affected by the salinization of their habitats, we need to know whether the effects of salinized environments interact with biotic stressors in ways that are additive, synergistic, or antagonistic.

To address these questions, we examined how varying concentrations of 2 different types of road salt affected amphibian sex ratios and whether these effects were altered in the presence of natural stressors (i.e., no-stressor control, competition, predator cues). We hypothesized that exposure to road salts would induce a higher proportion of male frogs and that the addition of natural stressors (i.e., competition and predator cues) would exacerbate this effect.

MATERIALS AND METHODS

Outdoor mesocosms

We examined the sex ratio of wood frogs (*Rana sylvatica*) from a published outdoor mesocosm experiment (Jones et al. 2017). The experimental design consisted of 7 salt treatments: a no-salt-added control (40 mg Cl^-/L) and either NaCl or a salt mix (NaCl, $MgCl_2$, and potassium chloride [KCl]) at 3 nominal concentrations: low (200 mg Cl^-/L), medium (600 mg Cl^-/L), and high (1000 mg Cl^-/L). These 7 treatments were crossed with 3 natural biotic environments: 1) a no-stressor control, 2) competition, or 3) predator cues. The 21 treatment combinations were replicated 4 times, resulting in 84 experimental units. In the present study, we dissected the preserved metamorphs from Jones et al. (2017) to examine effects on amphibian sex ratios.

The experimental units were outdoor mesocosms containing 82 L of tap water (added on 17–18 May 2015), 5 g of rabbit chow (added on 19 May), 100 g of dried leaf litter (added on 19 May), and a 0.53-L aliquot of natural pond water containing zooplankton and algae (added on 20 May). After allowing the mesocosms to grow algae and zooplankton populations, we added stage 25 wood frog tadpoles. According to Gosner (1960), this developmental stage of anurans has distinct characteristics that can be easily identified, and the tadpoles can be safely handled; it has also been shown to be a vulnerable developmental stage (Stănescu et al. 2017). On 22 April 2015, the wood frogs were initially collected as freshly laid egg masses from a natural pond (in Rensselaer County, NY, USA). Due to the nature of the original experimental hypotheses, American toads (*Anaxyrus americanus*) were also added to the mesocosms after being collected as eggs on 4 May 2015, to resemble a natural assemblage of animals living in their typical habitat (Werner et al. 2007). On 3 June 2015, mesocosms assigned the no-stressor and predator-cue treatments received 10 tadpoles of each species, and mesocosms assigned the competition treatment received 20 tadpoles of each species. Thus, the experimental increase in tadpole density created a competitive environment.

Each mesocosm also contained a predator cage. In mesocosms assigned the predator-cue treatment, the cage contained a larval dragonfly (*Anax junius*)—a well-known predator of larval amphibians. Larval amphibians sense predators through chemical cues given off during predation events and can respond by altering their behavior, physiology, and morphology (Crane et al. 2017; Relyea 2018). For example, larval dragonflies release kairomones that trigger a response from the larval amphibians. The larval dragonflies were fed 300 ± standard error tadpole biomass 3 times/wk, and dragonflies were replaced if they expired or had not consumed the previous meal.

After the tadpoles were added, we applied the salt treatments. The concentrations were tested with a calibrated chloride probe on days 15 and 21, and it was found that the no-salt-added concentration for the control was 21 mg Cl^-/L, the concentration for the low treatment group was 151 mg Cl^-/L, that for the medium group was 459 mg Cl^-/L, and that for the high group was 775 mg Cl^-/L. The tadpoles were distinguished as metamorphs by having 4 legs and any remnants of a tail. Once this transformation was observed, the animals were removed from the mesocosms and placed into separate containers until their tails were completely resorbed. They were then euthanized via an overdose of MS-222 and preserved in 10% formaldehyde. The last tadpole was removed on day 49. Further details can be found in Jones et al. (2016).

Wood frog sex determination

Using the preserved metamorphs, we dissected each frog to determine its sex. In wood frogs, sex can be reliably determined in newly metamorphosed individuals

(Lambert et al. 2016). The sex of each frog was determined by identifying the sexual organs, which were located on top of the kidneys. An ovary appears as a gelatinous material that is much larger than a testis. The testes are more uniform in structure, with a white tint, and are oval shaped. Dissections were conducted without any knowledge of the treatment.

Statistical analysis

Our analyses were conducted in R Ver 3.6.3 (R Development Core Team 2020). To determine the effect of salt concentration on sex ratios in the 3 different environments, we analyzed the NaCl and salt-mixture treatments separately, with proportion of females as our response variable. For the analysis of each salt type, we used a generalized linear model (glm) with a binomial error distribution and logit link function. Salt concentration, environment, and their interaction were included as predictor variables. Models for each salt type included the same no-salt controls within each biotic-stressor environment. The response variable was the proportion female, weighted by the total number of individuals in each sample. When we found significant ($p < 0.05$) or marginally significant ($p < 0.1$) effects in the glm, we performed an analysis of deviance using likelihood ratio tests to determine which predictor variables (salt type, salt concentration, or biotic stressor) explained a significant amount of the variability in sex ratios.

RESULTS

For NaCl treatments, the glm found no effect of concentration on the proportion of female frogs, and there were no interactive effects with the biotic stressors (Figure 1). The analysis of deviance further confirmed that including salt concentration, environment, or the interaction did not improve the model fit. Because there were no significant effects, we did not explore these effects further.

For salt mixture treatments, the glm found marginally significant effects of high concentrations in the no-stressor control environment, and a marginally significant interaction with the high concentration and predator environment (Figure 1). Although not statistically significant, these differences may still have ecological implications. Based on an analysis of deviance (Table 1), we found that including environment and its interaction with salt concentration did not significantly reduce model deviance ($p = 0.93$ and $p = 0.16$, respectively). Therefore, we refit the glm using only salt concentration as a predictor. When we combined these data across environments we found that the intermediate salt concentration caused the proportion of female frogs to decline by 15% ($p = 0.009$; Figure 2). Although we did not find a significant interaction between the intermediate salt concentration and biotic stressors, the intermediate concentration of the salt mixture caused a 14% decline in females with no stressor, an 11% decline in females with competitors, and a 23% decline in females with predator cues.

DISCUSSION

We sought to investigate the potentially interactive effects of road salt exposure and biotic stressors on the sex determination of amphibians. Biotic stressors alone did not induce a change in sex ratios, which was expected due to the lack of

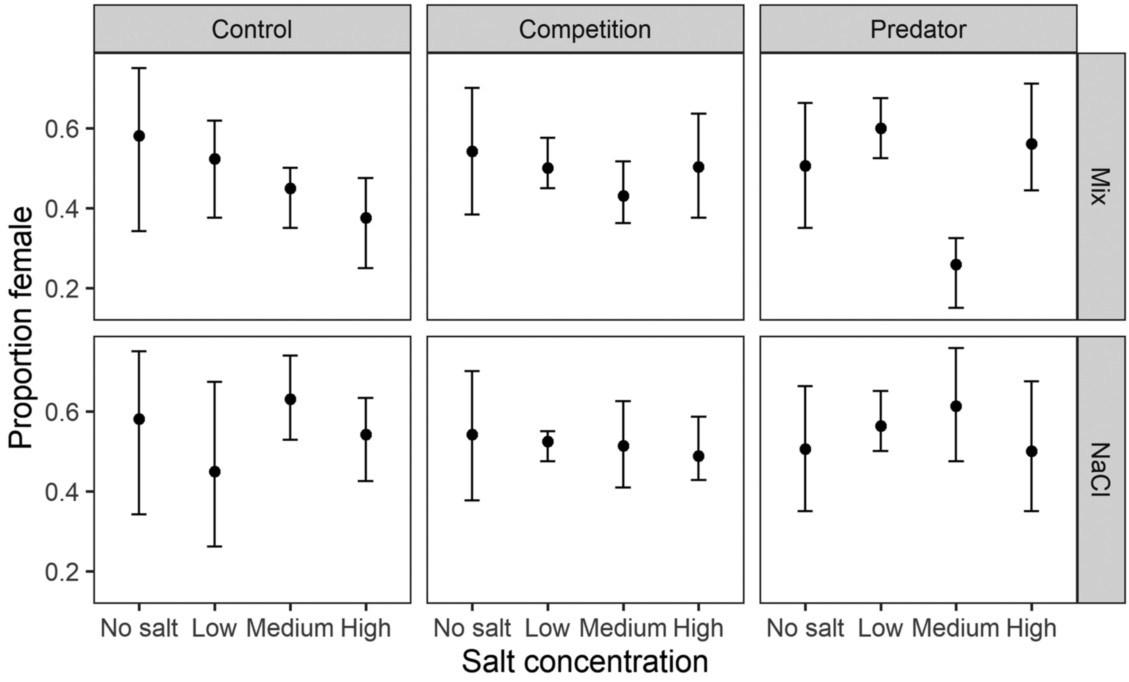

FIGURE 1: The proportion of female frogs metamorphosing from larval environments containing either a mixed-salt treatment (top) or NaCl treatment (bottom), crossed with either a control, competition, or predator environment. Note that the no-salt controls are the same for the top and bottom panels. Each point represents the mean of 4 replicates with bars representing the bootstrapped 95% confidence intervals.

TABLE 1: Results of the analysis of deviance for the generalized linear model (glm) on the effects of salt-mixture concentrations and biotic stressors experienced during the larval stage on the proportion of female frogs[a]

Salt mixture model	*df*	Deviance	Residual *df*	Residual deviance	*p* value
Intercept			47	61.5	
Concentration	3	8.76	44	52.7	0.03
Environment	2	0.14	42	52.6	0.93
Concentration:Environment	6	9.22	36	43.3	0.16

[a] Terms were added sequentially to the model (first to last) and compared using likelihood ratio tests.
df = degree of freedom.

association between competition and predation and sexual development in wood frogs. We also found that larval exposures to different concentrations of NaCl and the various biotic stressors did not alter sex ratios in metamorphic wood frogs. However, we discovered that exposure to the intermediate concentration (470 mg Cl^-/L) of the salt mixture caused 15% more males than expected in the population; the 54:46 sex ratio was shifted to a salt-induced 39:61 female:male sex ratio. Although we did not find that competitor or predator stress interacted with the road salts to affect the sex ratios, the masculinization was particularly strong when the medium salt mixture was combined with predator cues.

Whereas other contaminants such as herbicides can cause feminization in vertebrates, we know much less about the ability of salts to cause masculinization (Rohr and McCoy 2010). To our knowledge, only one previous study has examined the effects of road salt on amphibian sex ratios. Lambert et al. (2016) examined the effects of one concentration (867 mg Cl^-/L) of NaCl on the sex ratios of wood frogs compared with a low-salt control (114 mg Cl^-/L). They found that this high concentration had a masculinizing effect on wood frog populations, but they also noted that other salt concentrations should be examined in future studies. The highest concentration we tested (775 mg Cl^-/L) did not follow the same trend as Lambert et al. (2016), resulting in very little change in sex ratios. This discrepancy could be attributed to a salt-induction threshold that wood frogs may have developed to overcome extreme chemical changes in their environment. It may be that a larger chloride concentration is necessary to trigger the sex change. Our results at lower NaCl concentrations (150 mg Cl^-/L) did not induce a sex ratio change either, suggesting that such an induction may be very sensitive to NaCl concentration. However, the mixture of NaCl, $MgCl_2$, and KCl at an intermediate concentration (470 mg Cl^-/L) did induce a sex ratio change, which suggests that the $MgCl_2$ and KCl may play a key role in driving this effect. Magnesium and potassium may be amplifying the absorption of chloride, thus exacerbating its effect on sex development.

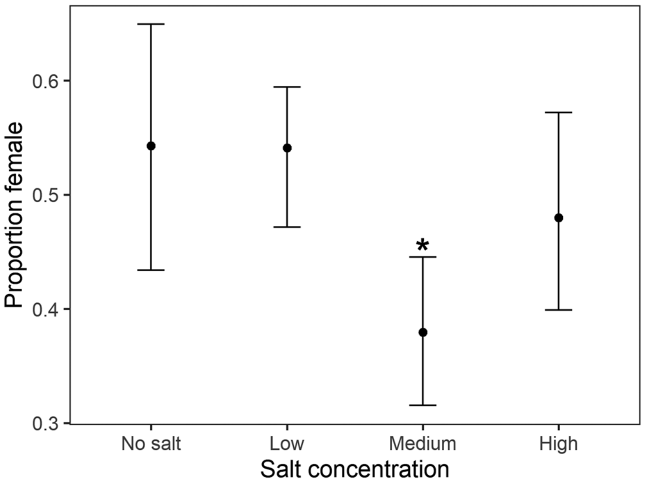

FIGURE 2: The proportion of female frogs metamorphosing from larval environments containing a salt mixture at different concentrations. Each point represents the mean of 12 replicates (pooled across all 3 biotic stressor environments) with bars representing the bootstrapped 95% confidence intervals.

The observation of salt-induced sex changes in wood frogs based on the present study and that of Lambert et al. (2016) suggests that other species of amphibians may also be susceptible. Given that aquatic habitats can experience a wide range of salt concentrations, from 0 to 13 500 mg Cl^-/L (Holmstrup et al. 2010), a considerable number of species might experience elevated chloride concentrations. Until we understand the underlying mechanism for this induction of more males in a population, it remains unclear whether other aquatic species (e.g., fish, macroinvertebrates) might also experience salt-induced changes in their sex ratios.

According to the salt-induced sex ratios in both our study and that of Lambert et al. (2016), there was an increase in the percentage of male frogs produced. Reducing the number of females in a population can be harmful to amphibian populations (Grayson et al. 2014). Most species have a balanced sex ratio as a result of frequency-dependent selection, such that there are fitness benefits of producing the less common sex in a population (Relyea and Ricklefs 2018). Thus, shifting a sex ratio toward more males might threaten the future growth of a population. This is certainly the case for amphibians, because one male can fertilize multiple females (Howard 1980; Berven 1981). As a result, the number of clutches deposited in a pond or wetland each year is a function of how many females are present. All things being equal, multiple years of a wetland experiencing a relatively high salt concentration (e.g., the medium concentration in our study, 470 mg Cl^-/L) could result in multiple cohorts of tadpoles being 35% female rather than an estimated 50% female; this would result in approximately one-third fewer females in the population. This, in turn, would result in approximately one-third fewer egg masses being laid. Whether it affects the number of metamorphs emerging would depend on the strength of density-dependence factors.

Moving forward, it would be beneficial to look at how different density-dependence factors can affect the way that varying road salt concentrations can alter sex ratios of amphibians. Factors such as the availability of resources or space may also impact the effects of road salt. Combining all of these factors will provide a better understanding of the potential damage road salt could have on the population dynamics of wood frogs and possibly other species.

CONCLUSIONS

Because society remains dependent on salt applications for safe roads, driveways, and parking lots, we need to consider the lethal and sublethal effects of these salts entering aquatic ecosystems. We need to understand whether the salt-induced sex ratios observed in wood frogs can occur in other species of amphibians as well as other aquatic species, using controlled experiments. If so, then we need to determine whether we find similar altered sex ratios in a more natural environment. We also need to identify the underlying mechanisms for the sex change and why (in our study) the sex change occurred at medium concentrations of the salt mixture, but not at higher and lower concentrations. We also need to understand how other salt formulations affect sex ratios, including formulations that are becoming increasingly popular, such as $MgCl_2$, $CaCl_2$, and KCl.

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Data Availability Statement—Data, associated metadata, and calculation tools are available from the corresponding author (legges2@rpi.edu). The data that support the findings of the present study are available on the EDI Data Portal by visiting https://portal.edirepository.org/nis/mapbrowse?packageid=edi.645.1.

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