

# Interactive effects of road salt and leaf litter on wood frog sex ratios and sexual size dimorphism

Max R. Lambert, Aaron B. Stoler, Meredith S. Smylie, Rick A. Relyea, and David K. Skelly

**Abstract:** Myriad natural and anthropogenic chemicals alter aquatic vertebrate sex ratios, with implications for population dynamics. Despite 22 million metric tons of salt applied to US roads annually, with much of it entering aquatic environments, it is unknown whether salt impacts sex ratios. Moreover, changes in forest composition co-occur with increased road salt application, dramatically changing ecosystems. We explore how road salt (sodium chloride) and two leaf litter types might influence amphibian development. By examining wood frog (*Rana sylvatica* = *Lithobates sylvaticus*) metamorphs reared with different combinations of salt (114 and 867 mg Cl-L<sup>-1</sup>) and litter species (none, maple (*Acer rubrum*), oak (*Quercus* spp.)), we show that salt masculinizes tadpole sex ratios, whereas oak, but not maple, litter feminizes populations. Road salt addition eliminates sexual dimorphism in oak-reared tadpoles, but enhances sexual size dimorphisms in maple-reared tadpoles, producing larger females. We are the first to show that road salt and native tree leaf litter manipulates vertebrate sex ratios and sex-specific development. Human land use might therefore influence vertebrate development through direct effects of contamination and indirect effects of altered botanical composition.

**Résumé :** Une myriade de substances chimiques naturelles et d'origine humaine modifient les taux de masculinité chez les vertébrés aquatiques, ce qui a des conséquences sur la dynamique des populations. Malgré le fait que 22 millions de tonnes métriques de sel soient épanchées annuellement sur les routes des États-Unis, dont une bonne partie aboutit dans des milieux aquatiques, il n'est pas établi si ce sel a ou non des impacts sur les taux de masculinité. En outre, des changements à la composition des forêts ont lieu en même temps que l'augmentation de l'épandage de sel, entraînant des changements marqués aux écosystèmes. Nous explorons l'influence possible du sel de voirie (chlorure de sodium) et de deux types de litière de feuilles mortes sur le développement d'amphibiens. En examinant des métamorphes de grenouille des bois (*Rana sylvatica* = *Lithobates sylvaticus*) élevés en présence de différentes combinaisons de sel (114 et 867 mg Cl-L<sup>-1</sup>) et d'espèces de feuilles mortes (aucune, érable (*Acer rubrum*), chêne (*Quercus* spp.)), nous démontrons que le sel accroît le taux de masculinité des têtards alors que la litière de chêne, mais pas celle d'érable, féminise les populations. L'ajout de sel de voirie élimine le dimorphisme sexuel chez les têtards élevés en présence de litière de chêne, mais accroît le dimorphisme sexuel de la taille chez les têtards élevés en présence de litière d'érable, produisant des femelles plus grandes. Il s'agit de la première étude à démontrer que le sel de voirie et la litière de feuilles mortes modifient les taux de masculinité et le développement selon le sexe de vertébrés. L'utilisation du sol par les humains peut donc influencer le développement des vertébrés par l'entremise des effets directs de la contamination et des effets indirects de la modification de la composition des végétaux. [Traduit par la Rédaction]

## Introduction

Anthropogenic changes to natural habitats are frequently coupled with changes in organic and inorganic chemical loading in ecosystems. For example, suburban ponds are increasingly contaminated with a milieu of trace elements from runoff as well as organic chemicals from septic systems and lawns (Lambert et al. 2015). Many organic chemicals alter hormonal activity in vertebrates, subsequently influencing sexual development (Kloas et al. 2009; Lambert and Skelly 2016). Experiments have assessed the effects of numerous individual chemical contaminants (e.g., pesticides) on vertebrate development, often showing deviant gonad structures and sex ratios (Kloas et al. 2009). However, less attention has been given to the hormonal effects of inorganic contaminants or the effects of natural chemical sources (e.g., from plant matter) that might change concurrently with human land use. Evidence from in vitro breast cancer cell lines shows that inorganic trace elements, including a diversity of salts, can interact with sex hormone pathways in similar ways as more commonly

considered organic chemicals (Choe et al. 2003). Furthermore, recent work shows that feminization of wild frog populations is partly associated with increasing concentrations of inorganic chemicals (e.g., magnesium) with known estrogenic properties (Lambert et al. 2015). However, little experimental work has assessed whether inorganic chemicals can impact sex hormone pathways or sexual development in vertebrates. One set of inorganic contaminants of growing concern is road salts, which is increasingly used as a deicing agent. Annually, over 22 million metric tons of road salt is currently applied to roads in the US (Findlay and Kelly 2011). Runoff of salt from roads has led to substantial increases in chloride concentrations in freshwater systems, with diverse negative consequences for aquatic communities (Karraker et al. 2008; Van Meter et al. 2012; Brady 2013; Dananay et al. 2015). Although high levels (>1000 mg Cl-L<sup>-1</sup>) can be toxic to many organisms, sublethal effects of salt, including deviant sexual development, remain largely unexplored.

The same land use development (e.g., roads, commercial infrastructure, residential neighborhoods) that necessitates an in-

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M.R. Lambert, M.S. Smylie, and D.K. Skelly. School of Forestry and Environmental Studies, Yale University, New Haven, CT 06511, USA.

A.B. Stoler and R.A. Relyea. Department of Biological Sciences, Rensselaer Polytechnic Institute, Troy, NY 12180, USA.

Corresponding author: Max R. Lambert (email: [max.lambert@yale.edu](mailto:max.lambert@yale.edu)).

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crease in road salt also generates changes in the composition of native, terrestrial flora that provide organic resource subsidies to freshwater systems (Lambert et al. 2015; Stoler and Relyea 2016; A. Stoler, B. Hintz, D. Jones, L. Lind, M. Schuler, and R. Relyea, unpublished data). For example, changes in floral composition can occur through active landscaping practices (Lambert et al. 2015) or through increased herbivory, due to higher deer densities associated with human residences (Abrams 1998). Changing floral communities are important because there is growing interest in understanding whether plant-derived chemicals (i.e., phytochemicals) affect vertebrate sexual development (Hermelink et al. 2010; Kolpin et al. 2010). Various phytochemicals elicit estrogenic and androgenic effects (Bovee et al. 2008), and hormonally active phytochemicals are commonly found in waterways from agricultural and urban landscapes (Kolpin et al. 2010; Lambert et al. 2015). Recent work showed that red clover (*Trifolium pratense*) root exudates, which contain hormonally active phytochemicals, masculinized sex ratios of metamorphosing wood frogs (*Rana sylvatica* = *Lithobates sylvaticus*; Lambert 2015). Similarly, yeast bioassays show that English oak (*Quercus robur*) leaves exhibit slight anti-androgenic and estrogenic properties when leached over short (24 h) periods (Hermelink et al. 2010). While we know that some plant species can exert hormonal effects on developing wildlife, no studies, to our knowledge, have assessed the hormonal effects of dominant tree species in the northeastern US. Given ongoing declines and compositional shifts in forests around the globe (Crowther et al. 2015), there is an immediate need to further understand whether changing tree composition influences vertebrate sex.

Little attention has been given to the independent effects of inorganic chemicals or chemicals produced by plants on sexual development, and even less work has explored how these different chemical classes interact to alter sexual development and sexual dimorphisms (Kortenkamp 2007). This is problematic because most aquatic systems are subject to diverse contaminants simultaneously (Côte et al. 2016). Here, we examined whether road salt additions and leaf litter of two tree genera (red maple (*Acer rubrum*) and mixed oak (two *Quercus* spp.)) independently and interactively influence wood frog sex ratios and sexual size dimorphisms of wood frog metamorphs. Although salts are known to bind to estrogen receptors in *in vitro* cell lines (Choe et al. 2003), it is unclear whether salts have agonistic or antagonistic influences on the estrogen pathway. Because of this, we predicted that salt will influence sexual development in wood frogs but cannot predict the direction (feminizing or masculinizing) of this effect. On the other hand, because leaf litter — and particularly oak litter — shows estrogenic and anti-androgenic properties in *in vitro* yeast assays (Hermelink et al. 2010), we predicted that the presence of leaf litter would generally feminize wood frog sex ratios relative to no-litter treatments. To test these predictions, we capitalized on experimental wood frog specimens preserved from a prior study evaluating the effects of different leaf litter species and road salt on aquatic ecosystem properties (A. Stoler, B. Hintz, D. Jones, L. Lind, M. Schuler, and R. Relyea, unpublished data).

## Methods

### Experimental design

Wood frog specimens from this experiment are a subset used in a prior experiment that evaluated community-level responses to different leaf litter and road salt additions. The experimental design is explained in detail elsewhere (A. Stoler, B. Hintz, D. Jones, L. Lind, M. Schuler, and R. Relyea, unpublished data). Briefly, we conducted our experiment during summer 2015 at the Rensselaer Aquatic Lab in Troy, New York. The original experiment consisted of three leaf litter treatments (no litter, red maple, and a mixture of black oak (*Quercus velutina*) and red oak (*Quercus rubra*)) crossed with four salt treatments (additions of 0, 100, 200, and 800 mg Cl·L<sup>-1</sup>). Both tree genera are common and dominant

throughout temperate North America, but recent decades have seen an expansion of maple because of deer over-browsing on oak, logging, and fire suppression (Abrams 1998). Because our goal in this study was to explore the possible range of effects of road salt, we only assessed wood frog individuals from the two most disparate salt treatments (additions of 0 and 800 mg Cl·L<sup>-1</sup>). These two salt concentrations are representative of wetlands immediately adjacent to a treated roadway, as well as stormwater retention ponds (Environment Canada, 2001; Van Meter et al. 2012). We replicated each treatment four times. Our experimental units were 750 L plastic mesocosms positioned in a randomized grid array, filled with ~500 L of well water, and covered with 60% shade cloth. On 14 May, we added 20 g of rabbit chow to each mesocosm to simulate an initial nutrient and energy supply that would be present in natural systems after spring thaw. Three days after filling mesocosms, we analyzed the chloride concentration of the well water, which was 60 mg Cl·L<sup>-1</sup>. We verified chloride levels in our mesocosms on day 25 by taking measurements with a calibrated, handheld multimeter (YSI) just below the water surface and just above the leaf litter. On average, chloride concentrations in the ambient and 800 mg Cl·L<sup>-1</sup> treatments were 114 and 867 mg Cl·L<sup>-1</sup>, respectively.

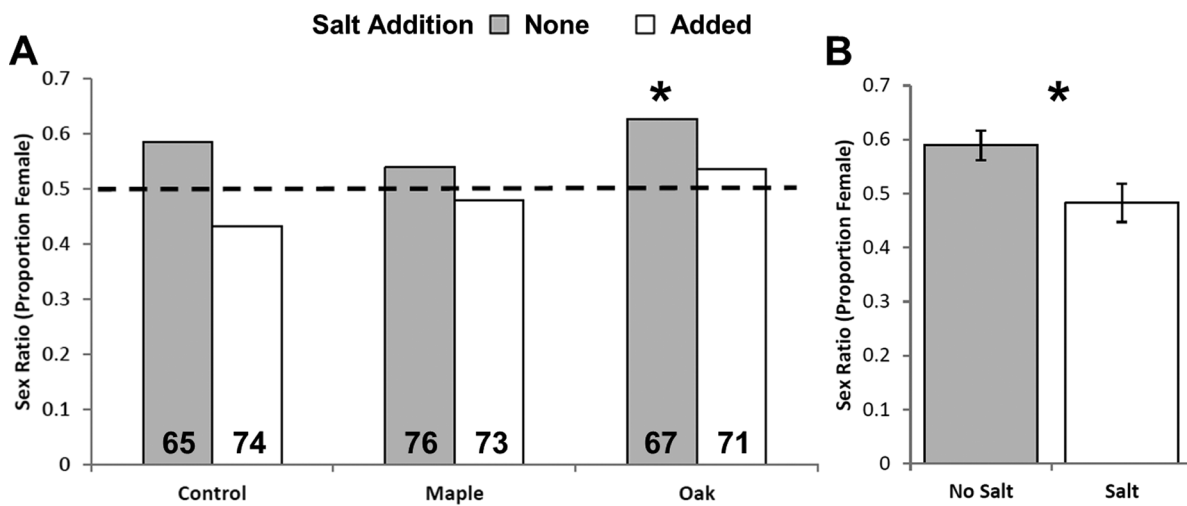
On 15 May, we added 250 g of dried leaf litter to all mesocosms, except for those mesocosms assigned to the no-litter treatment. We collected litter from local forests during spring 2015 and air-dried it for one week. These litter concentrations mimic natural litter densities in forest ponds (Rubbo et al. 2008; Stoler et al. 2016). On the same day, we added 0.5 L of pond water to each mesocosm. This inoculation was a mixture of water collected from four local ponds and served as a source bacteria, fungi, algae, and zooplankton. All four ponds were moderately canopy-covered with tree species that included either maple or oak.

We added several larger consumers to mesocosms, including American toad tadpoles (*Anaxyrus americanus*, formerly *Bufo americanus*), wood frog tadpoles, and *Physella acuta* snails. We collected 10 wood frog egg masses from a single, local wetland. We hatched the eggs and reared the tadpoles in outdoor, common garden conditions with well water and rabbit chow *ad libitum* as a food resource. Once tadpoles reached Gosner stage 25 (Gosner 1960), we added 20 individuals to each mesocosm on 28 May. American toad tadpoles weighed 40 ± 26 mg ( $\mu \pm$  SE), and wood frog tadpoles weighed 122 ± 62 mg. We set aside 20 tadpoles to assess 24 h survival because of handling effects, which was 100%. On the same day, we added nine adult snails to each mesocosm; the snails had also been collected from a single wetland.

After establishing the community in each mesocosm, we added NaCl road salt (Ice-a-Away Rock Salt Ice Melter, Compass Minerals; 100% NaCl) to the mesocosms on 30 May, 2 days after establishing the community. We calculated the amount of salt to achieve our nominal chloride concentrations (see above) for resulting concentrations of 60 and 860 mg·L<sup>-1</sup>. We added salt by removing 20 L of water from each mesocosm, dissolving the salt into the removed water, and then slowly pouring the water back into the mesocosm. To ensure that the salt dissolved evenly throughout the mesocosms, we distributed the water across the surface of the mesocosm and then gently stirred the mesocosm for approximately 10 s without disturbing the leaf litter. We repeated this process for ambient salt treatment, which had no salt addition, to equalize the disturbance across all treatments. We designated the day of salt addition as day 0 of the experiment.

In the days before wood frog metamorphosis began, we placed floating lath wood in all mesocosms as a substrate for metamorphs to climb upon. Upon collecting a metamorph, we held the froglet in the lab until its tail resorbed to <2 mm. We anesthetized all metamorphs in a 2% MS-222 solution and preserved them in 10% formalin. We measured snout-vent length (SVL) of each preserved metamorph and determined sex by inspection of gross gonadal morphology (see Lambert 2015; Warne and Crespi 2015).

**Fig. 1.** (A) Sex ratios of wood frog metamorphs reared without leaf litter (Control) or in the presence of maple or oak leaf litter and exposed to either ambient salt or an additional 800 mg Cl-L<sup>-1</sup>. Only metamorphs reared in oak litter without salt showed female-biased sex ratios (asterisk). Sex ratios did not differ across leaf litter treatments. The dashed line indicates equal sex ratios. Numbers at the base of each bar indicate the number of metamorphs in each sample. (B) Salt systematically reduced the proportion of females wood frog metamorphs (asterisk signifies significance;  $p \leq 0.05$ ).



Because toads (family Bufonidae) do not sexually differentiate until well after metamorphosis (Ogielska and Kotusz 2004), we did not assess toad sex ratios and sexual size dimorphisms in this study.

**Sex ratios**

To test whether sex ratios deviated from a hypothetical 50:50 ratio as is expected under normal sex chromosome segregation during meiosis, we performed chi-square ( $\chi^2$ ) tests. This statistical methodology is commonly used among sex ratio studies (Conover and Kynard 1981; Warner and Shine 2005). For this analysis we pooled samples within a treatment to increase power and minimize type 1 errors. We note that wood frog survival did not differ among treatments (A. Stoler, B. Hintz, D. Jones, L. Lind, M. Schuler, and R. Relyea, unpublished data). However, our main focus was on whether sex ratios varied among the treatments, and so we used a generalized linear model (GLM) with a binomial distribution and logit link function to test whether sex ratios differed among leaf and salt treatments. We started with a model incorporating an interaction between leaf and salt treatments, iteratively removing nonsignificant terms in the model after testing each term’s significance with analysis of variance (ANOVA). If either leaf or salt treatments were significant, we conducted Tukey’s post hoc tests using the “glht” function in the R package “multcomp”. We calculated the final model’s deviance explained ( $D^2$ ), a measure analogous to an  $R^2$  for non-Gaussian models, following Guisan and Zimmermann (2000).

To estimate whether any sex ratio variation was due to sex reversal or sex-biased mortality, we used binomial GLMs following established methods in (Hardy 2002; Zuur et al. 2009). We assessed whether sex ratios in a given mesocosm varied as a function of the number of metamorphs surviving in each mesocosm. In our models, we treated each experimental mesocosms sex ratio as a replicate rather than analyzing the pooled sex ratio across all mesocosms within a treatment. Survival did not vary among treatment on average (A. Stoler, B. Hintz, D. Jones, L. Lind, M. Schuler, and R. Relyea, unpublished data). If sex reversal were occurring, we would not expect to see any relationship between survivorship and sex ratios. If sex-biased mortality were occurring, we would expect more biased ratios in mesocosms with higher mortality.

**Sex-specific SVL**

Our goal was to assess whether there was any sexual dimorphism in metamorphic body size (SVL) and whether size dimor-

**Table 1.** Proportion of wood frog larvae surviving to metamorphosis ( $\mu \pm SE$ ) across treatments.

	Ambient salt	Road salt additions
No litter	0.95±0.02	0.94±0.02
Maple	0.98±0.01	0.91±0.04
Oak	0.99±0.01	0.93±0.03

phisms varied with leaf and salt treatments. To do this, we subtracted the mean male SVL from the mean female SVL in each mesocosm. We then used ANOVA to test whether differences in mean SVL between the sexes differed among litter and salt treatments, treating each mesocosm as a replicate. Because we were interested predominantly in the influence of different leaf litter species, we further explored whether there was an interaction between salt and leaf litter type if we only focused on treatments where leaf litter was present. As a reference, we also tested whether sexual size dimorphism was influenced by salt in the treatments without leaf litter using ANOVA. To test whether any treatment differed from 0 (indicating no sexual dimorphism), we used one-sample *t* tests. For analyses, we used R software (version 3.2.2; R Core Team 2015).

**Results**

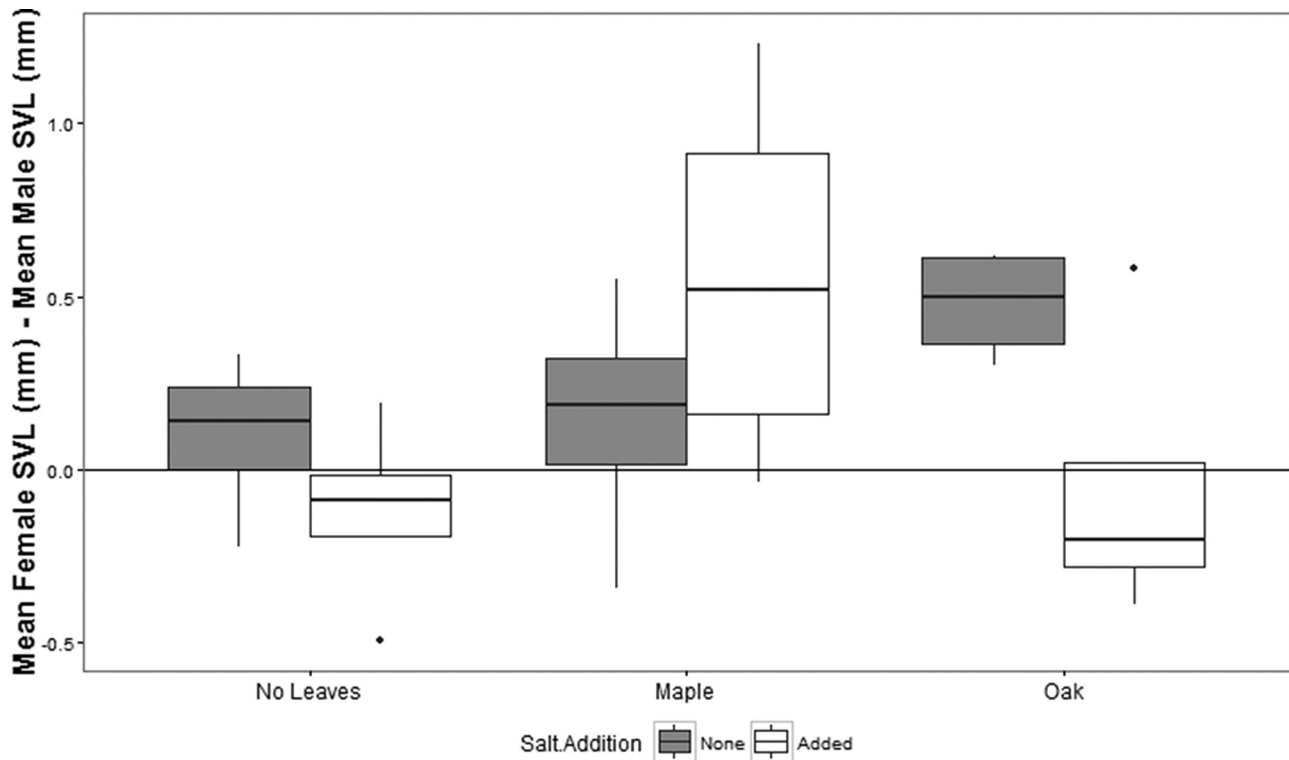
**Sex ratios**

Metamorphs reared in oak leaf litter in the absence of salt (Fig. 1A) exhibited a female-biased sex ratio (63% female,  $\chi^2 = 4.313$ ,  $p = 0.04$ ). Sex ratios in all other treatments contained statistically equal proportions of females and males (all  $p > 0.10$ ).

In the binomial glm, there was no interaction between leaf treatment and salt treatment on wood frog sex ratios ( $p = 0.73$ ), so we removed the interaction from the model. Similarly, sex ratios did not differ as a function of leaf litter ( $p = 0.36$ ), and we removed leaf litter treatment from the model. Sex ratios did, however, shift in response to salt ( $p = 0.04$ ,  $D^2 = 0.13$ ) such that salt exposure decreased the proportion of females in sex ratios (Fig. 1B).

Wood frog mortality did not differ between treatments (see A. Stoler, B. Hintz, D. Jones, L. Lind, M. Schuler, and R. Relyea, unpublished data; ANOVA  $F_{[2,36]} = 0.8$ ,  $p = 0.464$ ; Table 1 in the current article). Mesocosm sex ratios were not correlated with survivorship in any treatment (all  $p > 0.05$ ), indicating that sex

**Fig. 2.** Differences in body size (snout-vent length; SVL) between females and males across leaf litter and salt treatments. Without leaf litter, metamorphs show no sexual dimorphism, regardless of salt additions. Maple-reared metamorphs show enhanced sexual dimorphism with road salt additions. Under ambient salt, oak-reared metamorphs show sexual dimorphism, but this dimorphism is lost with the addition of road salt. The horizontal line crossing through 0.0 on the y axis indicates no sexual dimorphism, and positive values indicate females are larger than males. Boxes represent the first and third quartiles, while the vertical lines are the highest and lowest value within the interquartile range. Bold lines are the median, while black dots are outliers.



ratio variation is likely due to sex reversal rather than sex-biased mortality.

**SVL**

When analyzing SVL values with all three litter treatments, we found a marginally significant interaction between litter type and salt on sexual size dimorphism ( $F_{[2,18]} = 3.399, p = 0.056$ ), but no main effects of salt additions or litter type were not significant (both  $p > 0.1$ ). When we focused only on treatments containing leaf litter, we found a significant interaction between leaf litter type and salt ( $F_{[1,12]} = 5.249, p = 0.04$ ) on metamorphic sexual size dimorphism (Fig. 2). The main effects of litter and salt were not significant (both  $p > 0.1$ ). Results for the two litter species contrasted (Table 2). Among treatments with red maple litter, females metamorphosed 1.5% larger than males under ambient salt conditions; with road salt additions, females metamorphosed 1.9% times the size of males. Among treatments with oak litter, females reared under ambient salt conditions metamorphosed 3.2% larger than males, but the addition of salt reversed this dimorphism such that males metamorphosed 0.7% larger than females. Sexual size dimorphism was unaffected by salt additions in the treatments without leaf litter ( $F_{[1,6]} = 1.384, p = 0.28$ ). Only tadpoles reared with oak litter and ambient salt showed sexual size dimorphism ( $p = 0.009$ ), with females metamorphosing larger than males (Fig. 2). In all other treatments, there was no apparent sexual size dimorphism (all  $p > 0.1$ ).

**Discussion**

Salt decreased the proportion of female metamorphs by 10%, suggesting sodium chloride has a masculinizing effect (i.e., is anti-estrogenic). Sublethal salt contamination might therefore have

**Table 2.** Summary of metamorphic snout-vent length (mm;  $\mu \pm SE$ ) across sexes and treatments.

	Ambient salt		Road salt additions	
	Female	Male	Female	Male
No litter	11.89±0.22	11.91±0.19	13.11±0.23	13.11±0.21
Maple	15.61±0.14	15.38±0.15	15.58±0.13	15.28±0.19
Oak	15.46±0.13	14.96±0.12	14.99±0.12	15.09±0.17

effects on aquatic wildlife populations not previously considered. Two mechanisms can contribute to sex ratio variation: sex reversal and sex-biased mortality. While we cannot fully discount the role of sex-biased mortality with our present data, our results suggest that sex ratios did not vary with survivorship. This finding supports sex reversal as a likely explanation for the effect of salt on sex ratios. Further work, including developing sex-linked markers (Alho et al. 2010), is needed to more fully evaluate the contribution of sex reversal to these patterns.

Tadpoles reared in oak litter had feminized sex ratios, but only in the absence of salt. Although tadpole sex ratios from the oak treatment deviated from parity, we note that these sex ratios were statistically indistinguishable from sex ratios in the other treatments. Yeast assays have shown that leaf leachate of a European oak (*Q. robur*) is both estrogenic as well as anti-androgenic (Hermelink et al. 2010). However, the same study found no effect of leachate on tadpole sex ratios, whereas we found that oak produced a female bias. This discrepancy could be due to differences of litter chemistry and tadpole species between studies. For example, one of the oaks used here, *Q. rubra*, has lower lignin content and a relatively rapid decay rate (Hobbie et al. 2006) compared with the *Q. robur*

used by Hermelink et al. (2010). It could also be due to differences in experimental design; we exposed tadpoles to leaf litter that was continuously leaching throughout the experiment, whereas Hermelink et al. (2010) used short-term (24 h) leaf extracts. Regardless, these results provide evidence for at least a slight feminizing effect of *Quercus* leaves, but more work in this genus, and others, is certainly warranted. One likely reason for this phenomenon is the interaction of oak leachate compounds with vertebrate steroid receptors, likely initiating a hormonal cascade that causes sexual differentiation to reverse directions from the genetic sex (Hermelink et al. 2010).

We also found that salt additions and litter types interactively influence sexual size dimorphism at metamorphosis, eliminating size differences in oak-reared tadpoles and slightly increasing size differences in maple-reared tadpoles. Some phytochemicals (Ji et al. 2007) as well as chlorinated salts (Kloas et al. 2009) can interact with the thyroid, which regulates metabolism and growth. Sexual dimorphism variation might reflect conflicting interactions between litter-specific chemicals and chlorinated salts on thyroid receptors that differ between the sexes. Alternatively, litter chemicals and salt might change the relative metamorphic timing between the sexes. If this occurred, males would metamorphose faster under certain conditions, such as with oak litter but no additional road salt, providing females with more resources to grow larger during the rest of ontogeny. Although we cannot distinguish the mechanism of sex-specific timing of metamorphosis in our current study, clover root exudate was previously shown to accelerate male, but not female, metamorphosis (Lambert 2015). Additionally, trace elements are generally known to reduce frog metamorphic size (Snodgrass et al. 2004), but we still need a better understanding of sex-specific responses to inorganic contaminants and interactions with other chemicals.

Our data indicate that human-induced changes in aquatic chemistry through contamination and alteration of natural resources can have ecologically important consequences for offspring sex ratios and sex-specific development. Variation in offspring sex ratios, particularly by masculinizing sex reversal, should decrease population size and reduce population viability, regardless of whether sex-reversed individuals have impaired fitness (Cotton and Wedekind 2008). These potential effects of chemicals that are widely distributed in the environment provide a powerful motivation to better understand chemical mediation of the structure and dynamics of wild populations.

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