



Temperature Associated Breeding Phenology of Amphibian and its Impact on Offspring Size in Contaminants

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ABSTRACT

*In this paper we explored the Temperature Associated Breeding Phenology of Amphibian and its Impact on Offspring Size. Climate change-related increases in temperature variability have serious consequences for animal phenology throughout the world. Warmer winter temperatures, for example, may cause 'false springs' in breeding phenology across species, putting organisms at danger of freezing conditions during reproduction or critical early life stages. As human activities continue to intrude on natural ecosystems, it's crucial to think about how breeding phenology interacts with other anthropogenic stresses (e.g., pollutants). We evaluated growth; tolerance to a common wetland pollutant (NaCl); and the capacity of tadpoles to adapt to fatal NaCl exposure after sublethal exposure early in life using 14 populations of an ubiquitous amphibian (wood frog; *Rana sylvatica*). We compared these measures over two breeding seasons (2018 and 2019) and populations of tadpoles with different breeding phenology from their parents (earlier-versus later-breeding cohorts). Early-breeding cohorts finished breeding operations before a winter storm in both years, whereas later-breeding cohorts finished breeding activities after a winter storm. The freezing conditions experienced by later-breeding cohorts in 2018 were more severe in size and duration than those experienced in 2019. In 2018, the later-breeding cohort's kids were bigger but less tolerant to NaCl than the earlier-breeding cohort's offspring. Following sublethal exposure early in life, the children of the earlier-breeding cohort were also able to acclimatise to a lethal dose of NaCl, while the later-breeding group became less tolerant to NaCl after acclimation. We did not find any detrimental impacts of later breeding phenology on reactions to NaCl in 2019, the warmer of the two mating seasons. These findings indicate that phenological changes that expose mating frogs to freezing temperatures may have cascade effects on offspring mass and capacity to withstand subsequent stresses, but the intensity of the freeze event will most likely determine this.*

KEYWORD: Temperature, Breeding, Phenology, Amphibian, Impact, Offspring Size

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INTRODUCTION

Exposure to environmental pollutants also poses a danger to wildlife populations [1]. The salinization of freshwater systems, which has grown over the last few decades as a result of agricultural irrigation, coastal flooding, and the use of road salts, is a rising source of worry. Salts, unlike other pollutants, are preserved in the aquatic environment [21] and may build up over time. The anticipated impacts of global warming on wetland evaporation, where greater evaporation is predicted to decrease water volume, possibly raising salt concentrations in freshwater systems [20], exacerbates the problem. These problems are especially troublesome for spring-breeding frogs, who breed after spring rains, which may wash road salts deposited over the winter months into breeding habitats [4, exposing amphibians to higher salinities at various life stages [2]. Given the possibility of increasing freshwater salinization as a result of climate change, it's important to understand how temperature and salt levels combine to affect animal health.

In ecology, determining how species are impacted by human climate change is a critical task. Phenological changes in key life-history events linked to increasing average temperatures and increased temperature variability are one of the most visible ecological consequences of climate change [14]. Warming temperatures in temperate zones have resulted in early spring breeding and migratory activity during the 1960s [61]; this trend is consistent across taxa (e.g., marine creatures; amphibians – [24]; plants – [3]; birds; insects ; mammals). The dangers associated with 'false springs' [6] are one cost of such temperature-induced changes in the phenology of organisms. Annual reproductive events may be prematurely cued (i.e., a false spring) as a result of climate change, exposing breeding individuals and/or their young at critical early life stages to season-typical frost or freezing temperatures. False springs were

first discovered in our knowledge of the impacts of climate change on species phenology [13]. Since then, many reported cases of freeze-induced plant mortality and damage owing to early blossoming and shooting have been recorded (e.g., in the winters of 2007 [26] and 2010).

Amphibians were found to have the most advanced phenological changes of all the plant and animal species studied in a thorough meta-analysis. Amphibian spring activities have changed more than twice as early as those of trees, birds, or butterflies, and eight times as early as those of non-woody plants during the research period [50]. The most severe amphibian species showed early changes of approximately 30 days each decade. Those that bred the earliest (i.e., *Rana sylvatica*, *Pseudacris crucifer*) were also found to have the greatest climate-change related phenological changes toward even earlier breeding in a research on eastern North American frogs [24]. Early spring breeding frogs are likely to be affected directly by post-emergence freezing due to increased temperature fluctuation and related false spring occurrences. Despite the fact that temperate adult frogs have mechanisms to deal with freezing temperatures, repeated freeze-thaw cycles have physiological costs that may result in carryover effects that harm progeny. The study of possible phenological carryover effects has significant implications for amphibian population persistence in the face of climate change.

While salinization has been shown to have a detrimental effect on wildlife health, prior research has shown that wildlife populations may adapt to pollutant exposure. Wu et al. discovered that pre-acclimation of larval Indian rice frogs (*Fejervarya limnocharis*) in 7 g L⁻¹ saltwater for 48 hours improved their survival to an otherwise fatal dosage of saltwater at an 11 g L⁻¹ concentration. Aside from pollutant acclimatization, studies indicate that changing temperatures, such as those anticipated as a result of climate change, have a role in organisms' capacity to tolerate contamination exposure. Kimberly and Salice, for example, subjected *PhysPlomin* snails to two distinct temperature regimes (22 and 28 °C) and then evaluated their cadmium sensitivity. When snails were acclimated to either temperature alone, no changes in cadmium toxicity were discovered. Snail mortality rose significantly when snails habituated to one temperature were transferred to the other (i.e., 22 to 28 °C, or 28 to 22 °C) at the time of cadmium exposure. As a result, contaminant acclimatization and temperature fluctuation may both affect contaminant toxicity in animals. However, because temperature variability under climate change scenarios can also influence organisms' breeding phenology, it's important to understand not only how temperature influences contaminant tolerance acclimation, but also how breeding phenology interacts with temperature to influence organisms' ability to acclimate to contaminant exposure. Shifts in breeding phenology may lead to carry over effects that affect their offspring's capacity to tolerate or adapt to pollutants by possibly exposing adults to stressful freezing circumstances prior to breeding.

The aim of this research was to see how adult exposure to various temperature conditions owing to phenological variation in mating dates affects offspring development and performance in a common early spring-breeding amphibian (wood frog; *Rana sylvatica*) (baseline tolerance to NaCl and ability to acclimate to NaCl). We evaluated the development and performance of children from earlier-breeding parental cohorts vs. later-breeding parental cohorts across two breeding seasons. When compared to earlier-breeding parental cohorts, later-breeding parental cohorts undergo an extra freeze-thaw cycle before to breeding in both years. We anticipated that tadpoles of parents who delayed breeding in our study populations and experienced a winter storm would suffer carryover effects, making them smaller and less able to endure and adapt to NaCl exposure, since repeated freeze-thaw cycles have physiological costs.

MATERIAL AND METHODS

Animal collection

We compared wood frog tadpoles from populations that bred prior to freezing conditions (earlier breeding cohorts) vs. populations that bred after freezing conditions (later breeding cohorts) across two breeding seasons to see how differences in adult breeding phenology influences offspring growth and responses to NaCl (2018 and 2019). In 2018, we collected egg masses from seven wood frog populations that bred on April 6 (earlier cohort) and egg masses from seven distinct populations that bred following a winter storm on April 13 (later cohort) (late cohort; Fig. 1; Fig. 2; Supplementary Materials Table 1). In 2019, we collected egg masses from five populations of wood frogs that bred on March 30 (earlier cohort) and egg masses from each of five populations that bred on April 7 (later cohort) (late cohort; Fig. 1; Fig. 2; Supplementary Materials Table 1). All of the egg masses were obtained from populations in western Pennsylvania, the United States of America (Fig. 1; Supplementary Materials Table 1). Wood frog populations in this area have a mating window that starts in early March and lasts through April [25].

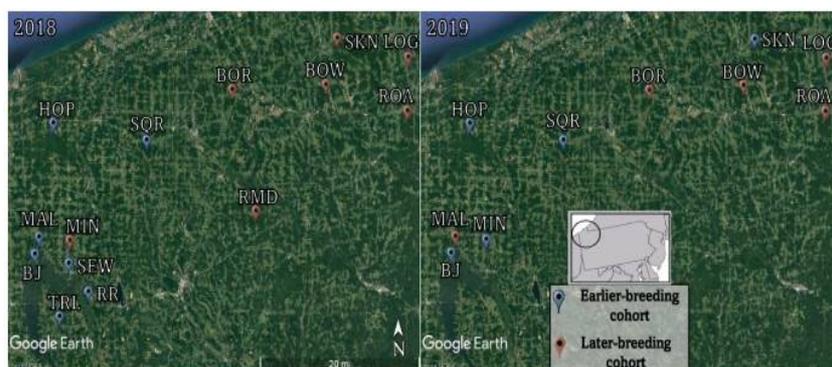


Figure 1 Map of wood frog source populations

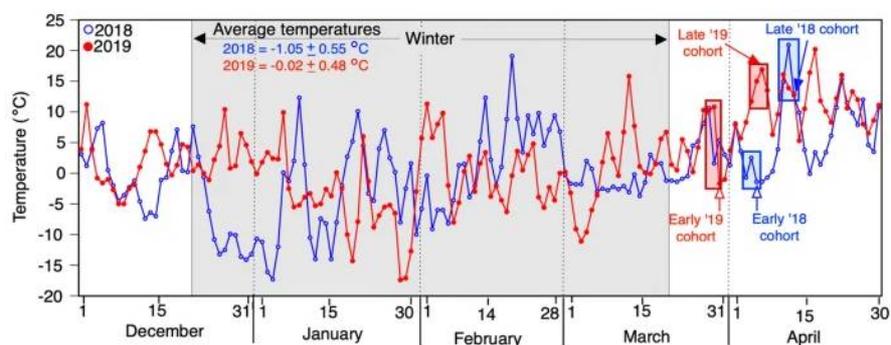


Figure 2 Average daily temperatures and wood frog egg collecting days for the early and late cohorts in 2018 (blue) and 2019 (red), respectively, at wood frog collection locations.

In both 2018 and 2019, we collected 10 incomplete egg masses from each location to guarantee a representative sample of each population. We collected egg masses within 48 hours after oviposition to reduce the impact of the environment on the egg masses.

- *Pond conductivity in the wild*

We assessed pond conductivity at the time of egg collection to account for variation in tadpole sensitivity to NaCl caused by variations in natal pond exposure to NaCl. A YSI Multiparameter Sonde was used to assess specific conductance (mean SE) in water samples collected from the edge and centre of each wetland (Xylem, OH, USA). Specific conductance is a measurement of water's capacity to transmit electrical current and is proportional to total ion concentration. As a result, our conductivity tests can't tell the difference between various salt ions in our water samples (e.g., chloride, sodium, magnesium, calcium, etc.). Specific conductance, on the other hand, has been found to be significantly linked with sodium and chloride ion concentrations in bodies of water, especially when road salts like NaCl are believed to be a major factor to conductance.

Design of an experiment

In Phase 1, we used a fully factorial design to place 50 embryos (Gosner stage 8; Gosner 1960) from each population in 2 L plastic tubs filled with 1.5 L of the appropriate Phase 1 pre-treatment solutions (Fig. 3). We assessed the mass of a selection of tadpoles (see Supplementary Materials Table 2 for sample sizes) from all populations across the three treatment doses after they reached the larval stage (Gosner stage 25). The time-to-death test was then started in Phase 2. (Hereafter TTD). All animals utilized in the mass measurements and TTD test reached Gosner stage 25 within 12 hours of one another across both years and cohorts. With a single tadpole/experimental unit, 100 mL cups were filled with 80 mL of either control or NaCl treatment water and were randomised across a single shelf unit. Between cohorts and years, all tests were conducted under similar temperature-controlled settings (20 °C).

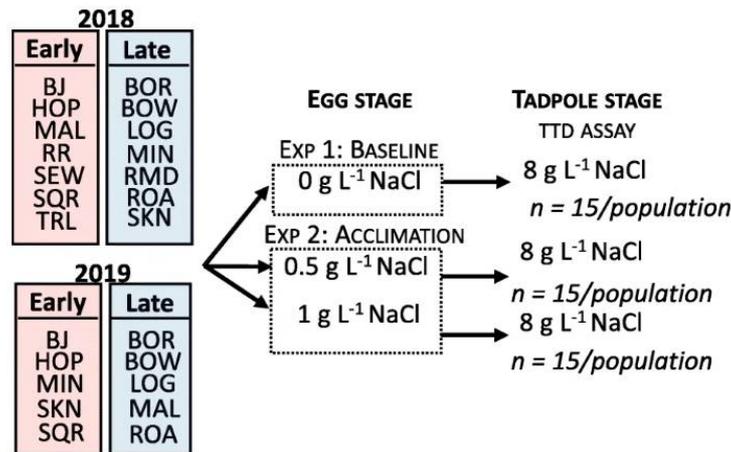


Figure 3 Schematic of the baseline and acclimated tolerance study design; The experimental strategy for determining the baseline tolerance and adapted tolerance of wood frogs to NaCl exposure is shown in this diagram.

We selected 8 g L⁻¹ NaCl, which has been proven to kill wood frog tadpoles [15]. Furthermore, 8 g L⁻¹ (4.85 g L⁻¹ Cl) is ecologically important, while being on the upper end of salt concentrations observed in natural systems, with urban ponds, streams, and rivers reaching concentrations of up to 13.50 g Cl L⁻¹ [20]. We assessed the TTD of all people by evaluating tadpole mortality every 2 hours until 100 percent mortality for information on the development of the Phase 1 and Phase 2 NaCl solutions. We did not feed any participants throughout the TTD test, as per normal toxicological practice. We utilized an overdose of unbuffered 5 percent MS-222 to euthanize all tadpoles that survived (control animals) and then stored all tadpoles used in the experiment in a 10% formalin solution.

- *Statistical analysis*
- *Pond conductivity in the wild*

To test the fixed effects of year, breeding cohort, and their interaction on pond conductivity, we employed a linear mixed effect model. We used modified Bonferroni testing to assess pairwise comparisons after finding significant main effects.

- *Gesner 25 has a tadpole mass.*

In 2018 and 2019, we utilized a linear mixed effect model to compare the mass of tadpoles from early vs. late cohorts after they were raised in different NaCl-acclimation treatments (0 g L⁻¹, 0.5 g L⁻¹, 1 g L⁻¹) in 2018. To account for variations in mass associated with development, we measured all tadpoles at Gesner stage 25. Our model included fixed factors for cohort, acclimation concentration, year, and their interactions, as well as a random variable for pond conductivity. Because the conductivity of the wood frog natal ponds varied, we incorporated it in our model (Fig. 4).

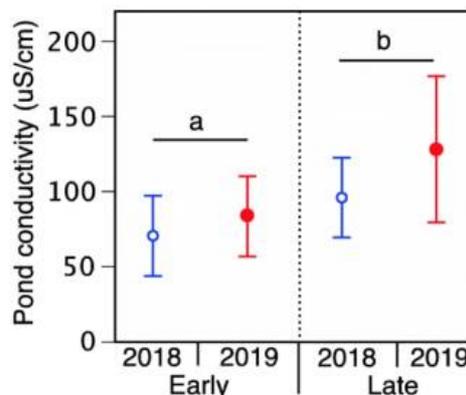


Figure 4 At wood frog locations, average pond conductivity (standard error); Natal Pond conductivity for early vs. late breeding cohorts in 2018 and 2019. At $p < 0.05$, different letters indicate significant

- *Tolerance at the start*

In 2018 and 2019, we utilized Cox Mixed Effect models to evaluate the survival rates of tadpoles from early vs. late cohorts. Only tadpoles raised in the 0 g L⁻¹ NaCl acclimation treatment were evaluated for baseline tolerance. We used cohort, year, and their interaction as fixed factors, and population and pond

conductivity as random effects in the first analysis. We performed two additional analyses, one for 2018 and one for 2019, since there was a substantial cohort*year interaction. R version 3.6 was used to conduct these analyses.

- *The impact of acclimatization on mortality*

The beta coefficient (β), a measure of change in mortality risk for tadpoles exposed to the acclimation treatments compared to a one-unit change in mortality rate of tadpoles exposed to the control, was calculated using Cox regression analysis. We computed a separate for each population in 2018 and 2019 by comparing the survival curves of tadpoles exposed to each acclimation treatment (0.5 g L⁻¹ and 1 g L⁻¹) to the survival curves of tadpoles from the same population who were not exposed to NaCl (0 g L⁻¹). Tadpoles subjected to the acclimatization therapy had a reduced risk of death than tadpoles not exposed to the acclimation treatment, as shown by a negative β -value. A positive β -value shows that tadpoles that received the acclimatization therapy had a greater death rate than tadpoles who did not receive the acclimation treatment. We used a linear mixed effect model to evaluate acclimation in tadpoles (β) from earlier and later cohorts exposed to different NaCl-acclimation treatments (0.5 g L⁻¹ vs. 1 g L⁻¹) in 2018 and 2019.

RESULTS AND DISCUSSIONS

- *Pond conductivity in the wild*

We observed no impact of year ($F_{1, 7.8} = 1.7$; $p = 0.23$) or breeding cohort*year interaction ($F_{1, 8.5} = 0.001$; $p = 0.98$) on natal pond conductivity using a linear mixed effect model. On the other hand, there was a significant main impact of breeding cohort on natal pond conductivity ($F_{1, 12.5} = 12.5$; $p = 0.013$). Pond conductivity was greater for populations of tadpoles from later breeding cohorts than it was for populations of tadpoles from earlier breeding cohorts (Fig. 4).

We used correlation analyses to see whether patterns of natal pond conductivity had an impact on tadpole tolerance to NaCl. For tadpoles raised in 0 g L⁻¹ ($r = 0.22$; $p = 0.31$), 0.5 g L⁻¹ ($r = 0.34$; $p = 0.12$), or 1 g L⁻¹ ($r = 0.18$; $p = 0.41$), there was no association between natal pond conductivity and tadpole tolerance to NaCl. While pond conductivity varies across cohorts, these differences do not seem to affect tadpole tolerance to NaCl, according to these findings. We do, however, add natal pond conductivity as a random factor in all studies as a precaution.

- *Gesner 25 has a tadpole mass.*

We discovered a significant main impact of cohort ($F_{1,21.3} = 5.0$; $p = 0.04$), NaCl-acclimation therapy ($F_{2,599.9} = 14.9$; $p = 0.001$), and year ($F_{1,11.5} = 27.6$; $p = 0.001$) on tadpole mass at Gesner stage 25 using a linear mixed effect model. While there was no significant interaction between Cohort and year ($F_{1,21.8} = 1.3$; $p = 0.25$), there were significant interactions between Cohort*NaCl-acclimation ($F_{2,600.1} = 10.2$; $p = 0.001$), Year*NaCl-acclimation ($F_{2,600.5} = 12.6$; $p = 0.001$), and Year*NaCl-acclimation*Cohort ($F_{1,600.5} = 6.6$; $p = 0.01$). We separately characterize the impact of earlier vs. late cohorts on tadpole mass at each year for each of the three NaCl-acclimation treatments to ease the explanation of significant interactions and in accordance with our a priori assumptions. We don't have data for tadpoles reared in a 0 g L⁻¹ environment for 2018, however there was no significant change in mass between early and late cohorts in 2019 ($p = 0.44$; Fig. 5). In 2018, there was no significant variation in mass between the early and late cohorts of tadpoles reared in 0.5 g L⁻¹ ($p = 0.44$; Fig. 5). In 2019, however, there was a statistically significant difference in mass between the early and late cohorts ($p = 0.04$; Fig. 5). Early breeding cohort tadpoles were smaller than later breeding cohort tadpoles. Finally, in both 2018 and 2019, there was a significant difference in mass between early and late cohorts for tadpoles reared in 1 g L⁻¹ ($p = 0.004$ and $p = 0.009$, respectively; Fig. 5).

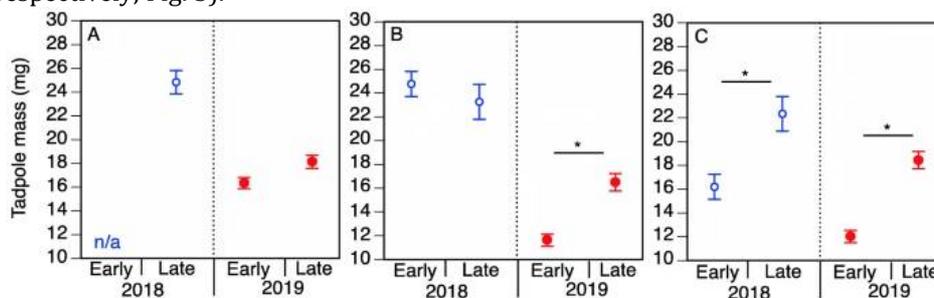


Figure 5 Early vs. late breeding cohorts in 2018 (blue) and 2019 (red) when pre-exposed to (a) 0 g L⁻¹ NaCl, (b) 0.5 g L⁻¹ NaCl, or (c) 1 g L⁻¹ NaCl; Average mass (standard error) of Gesner stage 25 tadpoles when pre-exposed to (a) 0 g L⁻¹ NaCl, (b) 0.5 g L⁻¹ NaCl. Significant differences ($p < 0.05$) between groups are indicated by asterisks (*).

While there was no impact of year ($\beta = 0.02$; $p = 0.9$) in the overall Cox Mixed Effect model, we did find a significant effect of cohort ($\beta = 5919.3$; $p = 0.001$) and cohort*year ($\beta = 2.9$; $p = 0.001$). We also ran two independent Cox Mixed Effects analysis for 2018 vs. 2019 to better understand the interaction. In 2018, we discovered a substantial cohort impact ($\beta = 4.4$; $p = 0.001$). Early breeding cohort tadpoles were considerably more tolerant to fatal NaCl concentrations than later breeding cohort tadpoles. In 2019, however, we did not detect a significant cohort impact ($\beta = 0.94$; $p = 0.7$; Fig. 6) in 2019.

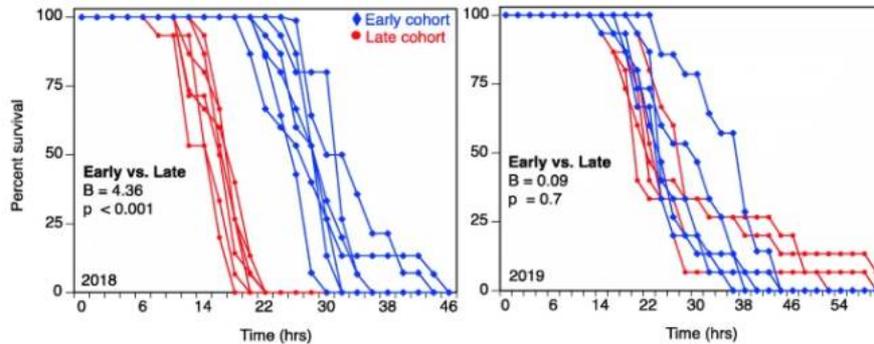


Figure 6 The effect of phenology on wood frog breeding NaCl tolerance; baseline tolerance to NaCl 8 g L⁻¹ NaCl of wood frog tadpoles from parents in earlier (blue) vs. late breeding (red) cohorts in 2018 and 2019.

The impact of acclimatization on mortality

We found no significant main effects of cohort ($F_{1,24} = 1.2$; $p = 0.28$), NaCl-acclimation treatment ($F_{1,24} = 1.2$; $p = 0.93$), or year ($F_{1,24} = 0.9$; $p = 0.35$) on tadpole acclimation (β) to NaCl using a linear mixed effect model (for details on (Beta coefficient) calculations and figures, see Supplementary Materials Figures a2, a3, a4). There was also no significant interaction between Cohort*NaCl-acclimation ($F_{1,24} = 1.2$; $p = 0.93$), Year*NaCl-acclimation ($F = 0.09$; $p = 0.76$), or Year*NaCl-acclimation*Cohort ($F_{1,24} = 1.1$; $p = 0.31$), or Year*NaCl-acclimation*Cohort ($F_{1,24} = 1.1$; $p = 0.31$). We did, however, discover a significant interaction between Cohort*year ($F_{1,24} = 4.8$; $p = 0.038$). We used Bonferroni-corrected paired comparisons to better understand this interaction. In 2018, we discovered that the capacity of tadpoles from earlier vs. later-breeding cohorts to adapt to increased NaCl concentrations was significantly affected by cohort ($F_{1,24} = 4.4$; $p = 0.001$). When compared to tadpoles from later breeding cohorts raised in either 0.5 g L⁻¹ or 1 g L⁻¹ NaCl, tadpoles from earlier breeding cohorts exhibited lower β -values (Fig. 7). This means that tadpoles from earlier breeding cohorts who were raised in 0.5 g L⁻¹ or 1 g L⁻¹ were more tolerant of higher NaCl concentrations (negative β), while tadpoles from later breeding cohorts were less tolerant of higher NaCl concentrations (positive β).

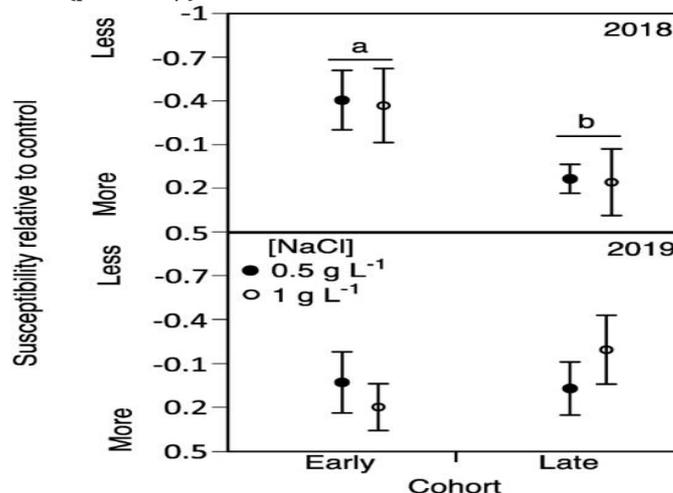


Figure 7 The effect of breeding phenology and NaCl exposure on acclimated tolerance in wood frogs; Wood frog tadpoles acclimated to NaCl (8 g L⁻¹ NaCl) from parents in earlier vs. late breeding cohorts in 2018 (top) and 2019 (bottom) after an acclimation phase in 0.5 g L⁻¹ or 1 g L⁻¹ NaCl. Statistical significance ($p < 0.05$) is shown by different letters above the groupings.

DISCUSSION

Anthropogenic activities have grave implications for the survival of species, especially those that are directly impacted by climate change and environmental pollutants. We looked at how a common spring-breeding frog is affected by breeding phenology (earlier vs later-breeding cohorts) and a common environmental contaminant. Several populations of *R. sylvatica* started mating early in response to spring-like temperature and precipitation signals in a single study area across two years; other populations did not breed until later, when the year's last severe winter weather events were finished. The earlier-breeding group generated smaller offspring than the later-breeding cohort in both years when raised in the highest NaCl acclimation treatment. Surprisingly, when NaCl levels in the rearing environment rose, the impact of breeding phenology on tadpole mass appeared to be worsened. Although we do not have data for the earlier cohorts of 2018, there is no change in mass between the early and late cohorts of 2019. The earlier cohort of 2019 generated substantially smaller individuals when raised at an intermediate concentration of NaCl (0.5 g L⁻¹) than the later-breeding cohort of the same year. Finally, children of the later-breeding cohorts of 2018 and 2019 weighed 37 percent and 40 percent higher on average than offspring of the earlier-breeding cohorts within their respective years when raised in the highest NaCl acclimation treatment (1 g L⁻¹ NaCl). This indicates that the interaction impact of phenology and NaCl on mass is likely dosage dependent, which is consistent with previous pollution studies [11].

We also discovered that wood frog tadpoles from the 2018 later-breeding cohort were less tolerant to NaCl than tadpoles from the same year's earlier-breeding cohort. While direct exposure to cold temperatures has been shown to suppress immune responses [16,], reduce larval growth and development, and interact synergistically with other stressors, it is important to note that tadpoles were not exposed to the cold temperatures of the parental environment in this study. As a result, the late breeding cohort's lower baseline tolerance to NaCl may represent cross-generational effects of parental exposure to suboptimal temperatures. For example, Piironen et al. discovered that cold stress caused deleterious cross-generational effects in Colorado potato beetles (*Leptinotarsa decemlineata*), with offspring of cold-stressed parents being less tolerant of pyrethroid insecticides than those whose parents were reared at more optimal temperatures. We anticipate animals to face increased temperature variability, which may affect breeding phenology, as well as difficulties associated with pollution exposure as a result of climate change. As a result, our results highlight the significance of assessing the effects of phenological changes in conjunction with additional stressors as NaCl exposure.

Some people may improve their future tolerance to pollutants after a period of acclimatization to sublethal doses of the pollutant to better deal with toxins in their environment. We show that certain populations of wood frogs may develop greater tolerance to NaCl after being exposed to it as embryos and hatchlings, which is consistent with previous research. Although earlier study has shown that wood frogs may improve pesticide tolerance, this is the first indication of NaCl acclimatization in this species to our knowledge. It's still unclear if this phenomenon can be applied to all toxicants. Though some data suggests that wood frog populations that can develop tolerance to one pollutant may also generate cross-tolerance other contaminants with comparable mechanisms of action. In 2018, the capacity of wood frogs to acclimatise to NaCl was shown to vary across early and later-breeding cohorts. In particular, children from the later-breeding cohort of 2018 whose parents encountered a freezing event before to breeding were less capable of acclimating to NaCl exposure than those from the earlier-breeding cohort of the same year whose parents did not. This trend was seen independently of the sublethal NaCl concentrations (0.5 or 1 g L⁻¹ NaCl) that tadpoles were exposed to prior to lethal exposure. While the reasons behind the later-breeding cohort's decreased capacity to adapt to NaCl exposure were outside the scope of this research, many studies have shown that amphibian acclimatization to salty environments is linked to changes in stress hormones such corticosterone (CORT). Extreme weather events, such as cold temperatures, have been shown to increase CORT production in animals [10,], which may subsequently be passed on to offspring [28]. While stress hormone expression helps to deal with short-term problems, persistently increased CORT has been linked to detrimental fitness consequences. Future research may look at whether the 2018 later-breeding cohort's decreased capacity to adapt to NaCl exposure is due to cross-generational fitness costs of parental stress hormone production. While there is considerable evidence that frogs may acclimatize to a variety of contaminants, our research indicates that determining the relative significance of acclimation as a strategy for population survival in the face of global change requires considering parental environmental circumstances.

We discovered that the impact of breeding phenology (earlier vs. later) on baseline tolerance and acclimation varied by year. The variations in NaCl baseline tolerance and acclimatization in 2018 and 2019 may be explained by temperature averages and fluctuation throughout the two winters of our research. In 2018, the later-breeding *R. sylvatica* cohort had a more severe winter weather event than the later-breeding *R. sylvatica* cohort in 2019 (5 vs 2 days of cold temperatures, respectively). Because there

are costly physiological responses to low temperatures [19], and maternal stress is often transferred to developing eggs [18,] in a variety of herpetofauna, the later-breeding cohort in 2018 likely experienced greater physiological stress from the freeze than the later-breeding cohort in 2019. One possible reason of decreased offspring baseline tolerance to NaCl in 2018 compared to 2019 is the greater degree of stress experienced by the later-breeding cohort. The difference in offspring NaCl tolerance (following NaCl acclimation) between earlier and later-breeding cohorts mirrors the baseline tolerance pattern: in 2018, the offspring of later breeders were more susceptible than those of earlier breeders, whereas in 2019, the offspring of earlier and later breeders were equally susceptible. Small changes in temperature in the maternal environment may have an effect on the survival of offspring in salinized systems, according to our results. Because shifting temperature regimes are expected to continue as a result of global climate change, resulting in increased NaCl pollution in some parts of the world [5] our findings point to the need for cross-generational studies to determine whether the patterns of earlier and later cohort survival following NaCl exposure seen here were caused by freezing temperatures in the parental environment.

Breeding time (earlier vs. later) does not seem to be a locally evolved feature in this system. Environmental signals, on the other hand, are well known to cause breeding behavior in wood frogs [7]. Wood frog breeding periods across populations are variable in the system we studied, frequently dividing into early vs. later breeding populations in certain breeding seasons (i.e., years with significant variability in environmental signals). In contrast, these identical wood frog populations may all spawn on the same day in other years. Variable breeding periods across and within wood frog populations throughout the years studied are consistent with other species, where differential breeding phenology has been attributed to microclimate and/or weather variations. Importantly, even within a population, there is evidence of yearly fluctuation in breeding time, as shown in this research. Indeed, between 2018 and 2019, wood frogs in the three populations (MIN, SKN, and MAL; Fig. 1) varied in whether they lay sooner or later. We were able to evaluate NaCl tolerance in early vs. later-breeding cohorts within a population because of this change in relative breeding phenology across years. Even when the population was held constant, we observed that tadpoles of earlier-breeders were more tolerant to NaCl than tadpoles of late-breeders, which was consistent with the general findings across populations (SI- Figure A10). This indicates that the early-late phenotypic variations in NaCl tolerance seen over the course of the two years of research are unlikely to be entirely attributable to underlying population-specific characteristics.

Finally, we discovered that the conductivity of natal pond locations chosen by the wood frog populations studied varied. The natal pond conductivity of certain frog species is linked to their resistance to NaCl exposure [8, 9, 30]. Our correlation studies, on the other hand, showed that natal pond conductivity was not a causative factor in the differences in NaCl tolerance seen in our study populations. Furthermore, since eggs were taken from natal ponds 48 hours after oviposition and promptly put in filtered well water, direct impacts of the natal pond environment on wood frog embryos were likely minimal. Given the absence of a link between pond conductivity and NaCl tolerance, as well as embryos' limited exposure to the natal environment, the primary contribution to the responses seen in our research seems to be variations in breeding phenology and/or parental environment temperature.

CONCLUSION

Our findings indicate that, when coupled with chemical stresses, freezes following false springs (exacerbated by higher average winter temperatures) are likely to be a significant issue for spring breeding amphibian populations. Despite some disagreements over whether climate change has a direct influence in amphibian extinctions [17], amphibians are becoming more recognized as extremely susceptible to climate change [23]. We believe that the interaction of climate-induced phenological changes and environmental pollutants poses a direct danger to amphibian populations that is understudied.

REFERENCES

1. Acevedo-Whitehouse K, Duffus ALJ. (2009). Effects of environmental change on wildlife health. *PhilosTrans R Soc Lond B Biol Sci.* ;364(1534):3429–38.
2. Albecker MA, McCoy MW. (2017). Adaptive responses to salinity stress across multiple life stages in anuran amphibians. *Front Zool.* 14(1):40.
3. Allstadt AJ, Vavrus SJ, Heglund PJ, Pidgeon AM, Thogmartin WE, Radeloff VC. (2015). Spring plant phenology and false springs in the conterminous US during the 21st century. *Environ Res Lett.* ;10(10):104008.
4. Andrews KM, Nanjappa P, Riley SPD. (2015). Roads and ecological infrastructure: concepts and applications for small animals. Baltimore: John's Hopkins University Press.

5. Arvidsson A, Blomqvist G, Öberg G. (2012). Impact of climate change on use of anti-icing and deicing salt in Sweden. In: Winter maintenance and surface transportation weather: international conference on winter maintenance and surface transportation weather, vol. 30. Coralville: IA p. 3–10.
6. Ault TR, Henebry GM, De Beurs KM, Schwartz MD, Betancourt JL, Moore D. (2013). The false spring of 2012, earliest in north American record. *Eos*. 94(20):181–2.
7. Benard MF. (2015). Warmer winters reduce frog fecundity and shift breeding phenology, which consequently alters larval development and metamorphic timing. *Glob Chang Biol*. 21(3):1058–65.
8. Brady SP. (2012). Road to evolution? Local adaptation to road adjacency in an amphibian (*Ambystoma maculatum*). *Sci Rep*. 2(1):235.
9. Brady SP. (2013). Microgeographic maladaptive performance and deme depression in response to roads and runoff. *PeerJ*. 1:e163.
10. Breuner CW, Wingfield JC, Romero LM. (1999). Diel rhythms of basal and stress-induced corticosterone in a wild, seasonal vertebrate, Gambel's white-crowned sparrow. *J Exp Zool*. 284(3):334–42.
11. Budischak SA, Belden LK, Hopkins WA. (2008). Effects of malathion on embryonic development and latent susceptibility to trematode parasites in ranid tadpoles. *Environ Toxicol Chem*. ;27(12):2496–500.
12. Cañedo-Argüelles M, Hawkins CP, Kefford BJ, Schäfer RB, Dyack BJ, Brucet S, et al. (2016). Saving freshwater from salts. *Science*. 351(6276):914–6. <https://doi.org/10.1126/science.aad3488>.
13. Cannell MGR, Smith RI. (1986). Climatic warming, spring budburst, and forest damage on trees. *J Appl Ecol*.;23(1):177–91.
14. Cohen JM, Lajeunesse MJ, Rohr JR. (2018). A global synthesis of animal phenological responses to climate change. *Nat Clim Chang*. 8(3):224–8.
15. Collins SJ, Russell RW. (2009). Toxicity of road salt to Nova Scotia amphibians. *Environ Pollut*. 157(1):320–4.
16. Cone RE, Marchalonis JJ. (1972). Cellular and humoral aspects of the influence of environmental temperature on the immune response of poikilothermic vertebrates. *J Immunol*. ;108(4):952–7.
17. Corn PS. (2005). Climate change and amphibians. *Anim Biodivers Conserv*. 28(1):59–67.
18. Cree A, Tyrrell CL, Preest MR, Thorburn D, Guillette LJ. (2003). Protecting embryos from stress: corticosterone effects and the corticosterone response to capture and confinement during pregnancy in a live-bearing lizard (*Hoplodactylus maculatus*). *Gen Comp Endocrinol*. 134(3):316–29.
19. Dupoué A, Brischoux F, Lourdais O, Angelier F. (2013). Influence of temperature on the corticosterone stress-response: an experiment in the children's python (*Antaresia childreni*). *Gen Comp Endocrinol*. 2013;193(2013):178–84. <https://doi.org/10.1016/j.ygcen.2013.08.004>.
20. Evans M, Frick C. (2001). The effects of road salts on aquatic ecosystems.
21. Findlay SEG, Kelly VR. (2011). Emerging indirect and long-term road salt effects on ecosystems. *Ann N Y Acad Sci*. 1223(1):58–68.
22. Flemming MH. (2015). PennDOT LTAP technical information sheet #167. Pennsylvania: Department of Transportation.
23. Foden WB, Butchart SHM, Stuart SN, Vié JC, Akçakaya HR, Angulo A, et al. (2013). Identifying the World's Most climate change vulnerable species: a systematic trait-based assessment of all birds, amphibians and corals. *PLoS One*. 8(6):e65427.
24. Gibbs JP, Breisch AR. (2001). Climate warming and calling phenology of frogs near Ithaca, New York, 1900–1999. *Conserv Biol*. 15(4):1175–8.
25. Gray BS, Lethaby M. (2008). The amphibians and reptiles of Erie County, Pennsylvania. *Bull Maryland Herpetol Soc*. 44(2):49–69.
26. Gu L, Hanson PJ, Post WM, Kaiser DP, Yang B, Nemani R, et al. (2007). The 2007 eastern US spring freeze: increased cold damage in a warming world? *BioScience*. 58(3):253–62.
27. Hall EM, Brady SP, Mattheus NM, Earley RL, Diamond M, Crespi EJ. (2017). Physiological consequences of exposure to salinized roadside ponds on wood frog larvae and adults. *Biol Conserv (Elsevier)*. 209:98–106.
28. Hau M, Casagrande S, Ouyang JQ, Baugh AT. (2016). Glucocorticoid-mediated phenotypes in vertebrates: multilevel variation and evolution. *Adv Study Behav*. 48:41–115.
29. Hintz WD, Relyea RA. (2019). A review of the species, community, and ecosystem impacts of road salt salinisation in fresh waters. *Freshw Biol*. 64(6):1081–97. <https://doi.org/10.1111/fwb.13286>.
30. Hopkins GR, Brodie ED Jr. (2015). Occurrence of amphibians in saline habitats: a review and evolutionary perspective. *Herpetol Monogr*. 2015;29(1):1–27.

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