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# Temperature variability and multiple environmental stressors: how will tadpole performance change with our climate?

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# Temperature variability and multiple environmental stressors: how will tadpole performance change with our climate?

Thesis by

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In partial fulfillment of the requirements for the University Scholars Program and the Degree of  
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The Effects of temperature variability on wood frog (*Lithobates sylvaticus*) and gray tree frog  
(*Hyla versicolor*) tadpole performance

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ABSTRACT.—Temperature is known to affect larval growth and development and may be the most influential environmental factor on larval amphibians. Climate change is predicted to increase not only mean temperatures but also temperature variability within a season. Here our goal was to examine how the growth and development of wood frog (*Lithobates sylvaticus*) and gray tree frog (*Hyla versicolor*) tadpoles are affected by temperature variability in the absence of food limitation, competition, and predation. While a number of studies have examined the impact of daily thermal fluctuations, our study focused on fluctuation frequencies pertaining to changing weather fronts and heat waves. We manipulated the frequency (once, weekly, or biweekly) and magnitude (4, 6, or 8 °C fluctuations) of temperature fluctuations for two randomized laboratory experiments in 2013 and an additional experiment in 2014. We found evidence of a developmental threshold for our experiment at a mean of 26 °C for *L. sylvaticus* tadpoles such that time to metamorphosis was reduced at this temperature, regardless of fluctuation. We found that within the (20–28 °C) range of elevated and fluctuating temperatures examined in this study, *H. versicolor* exhibited a reduced mass at metamorphosis relative to consistent weekly and biweekly temperature fluctuations (23 ± 3 °C) when exposed to a large 6 °C temperature fluctuation to 29 °C at the end of the larval period (but not at the beginning of the larval period). This result is consistent with the expectation of tadpoles metamorphosing as a wetland dries and demonstrates that temperature fluctuations early in the larval period have little influence on growth and development. *H. versicolor* also exhibited increased time to metamorphosis under biweekly temperature fluctuations relative to both high magnitude fluctuation treatments. We highlight the importance of incorporating temperature variability into amphibian research to improve our understanding of their responses to climate change.

*Key Words:* Climate change; thermal tolerance; amphibian; conservation, ectotherm



## INTRODUCTION

Amphibian susceptibility to ecosystem alteration has already caused amphibian populations to decline worldwide from habitat alterations, pollution, invasive species, and disease at rates higher than birds and mammals (IUCN et al., 2004), and the changing global climate poses a new threat to amphibian populations that may interact synergistically with other environmental stressors to adversely affect amphibian populations (Atkinson, 1996; Gibbs and Breisch, 2001; Parmesan and Yohe, 2003; Hickling et al., 2006; Klaus and Loughheed, 2013; Li et al. 2013; Rohr and Palmer, 2013). Predictive climate change models for the northeastern part of the United States show increases in average annual temperatures by as much as 3 °C by 2050 and almost 6 °C by 2100 (Hayhoe et al., 2008). Additionally, seasonally dependent changes to precipitation patterns could lead to water deficits in the summer (Hayhoe et al. 2008; Brooks, 2009). Summers in New England are expected to become warmer and experience more intense droughts, which could cause vernal pools to dry early and stay dry for longer periods of time, reducing successful amphibian reproduction through reduced larval fitness (Atkinson, 1996; Brooks, 2004; Hayhoe et al., 2008; Brooks, 2009; Gao et al., 2012; Salice, 2012; Rohr and Palmer, 2013). The predicted changes in hydrology could also alter community interactions and assemblages (Brooks, 2009). The changing global climate has already altered the phenology of behaviors such as spring emergence and breeding (Gibbs and Breisch, 2001; Todd et al., 2011; Klaus and Loughheed, 2013), and distributional shifts caused by changing climates combined with habitat alterations are likely to cause mismatched communities, collapsed distributions, and remnant populations of amphibians in the future (Parmesan and Yohe 2003; Hickling et al. 2006).

The behavior and development of ectothermic animals are particularly influenced by thermal regimes (Wilbur and Collins, 1973; Huey and Slatkin, 1976; Smith-Gill and Berven, 1979; Standora and Spotila, 1985; Shine, 2005; Tattersall and Ultsch, 2008; Walsh et al., 2008). Warm temperatures during anuran larval development typically induce physiological and morphological changes that cause faster growth and a smaller size at metamorphosis than cold temperatures (Smith-Gill and Berven, 1979; Walsh et al., 2008). Such responses to the thermal environment can increase tadpole survival because tadpoles must metamorphose early in situations with high temperatures and short hydroperiods or risk death (Werner, 1986; Rowe and Dunson, 1995, Atkinson, 1996; Newman, 1998; Laurila and Kujasalo, 1999; Ryan, 2007). Further, small differences in size at and time to metamorphosis can significantly affect the subsequent performance of the individual (Semlitsch et al., 1988; Touchon and Warkentin, 2010).

Despite the importance of the thermal environment to larval anuran development and the threat of climate change, most research analyzing the effects of climate change on amphibians focuses on predicted mean increases in temperature (Bustamante et al., 2010; Rohr and Palmer, 2013), which, although useful, does not accurately represent the temperature regime amphibians are subjected to in nature. Temperatures vary daily, weekly, and seasonally, and, with climate change, temperatures across these scales are expected to increase in variability (Ganguly et al., 2009; Gao et al., 2012; Christensen et al., 2013). Thus, in order to truly grasp the implications of climate change, temperature variability should be a fundamental aspect of amphibian research. A number of studies have examined the impact of daily thermal fluctuations on amphibians (Niehaus et al., 2006; Niehaus et al., 2011; Arrighi et al., 2013; Rojas et al., 2014; Kern et al.,

2015), but there is a need to better understand how other frequencies of thermal variability affect larval amphibian performance.

We sought to understand how fluctuation frequencies pertaining to changing weather fronts and heat waves affect the growth and development of larval *Lithobates (Rana) sylvaticus* (wood frog; LeConte, 1825; Frost et al., 2006) and *Hyla versicolor* (gray tree frog, LeConte, 1825). Thus, we performed two experiments in 2013 that varied in the frequency (once, weekly, or biweekly) and magnitude (max temperatures of 26 or 29 °C) of temperature fluctuation while maintaining the same mean temperature in all treatments, and we performed an additional experiment in 2014 that varied the mean (22, 24, or 26 °C) and magnitude (max temperatures of 24 or 28 °C) of temperature fluctuation. In our 2013 experiments, we predicted that wood frog and gray tree frog tadpoles reared in a fluctuating temperature environment would develop more quickly and have a smaller mass at metamorphosis compared to tadpoles raised at a stable temperature due to increased physiological stress (Manis and Claussen, 1986; John-Alder et al. 1988; Niehaus et al., 2006; Vasseur et al., 2014). Previous studies have found that tadpoles exposed to diurnal temperature fluctuations metamorphose earlier, at a smaller mass at metamorphosis, and with poorer body condition compared to tadpoles kept at a constant temperature (Niehaus et al., 2006; Kern et al., 2015). Similarly, we predicted that weekly temperature fluctuations would act more strongly as a stressor than biweekly temperature fluctuations, adversely affecting performance. We also predicted that a single, high magnitude temperature fluctuation to 29 °C over a week could result in early emergence, but only if the fluctuation occurs after the tadpoles have passed the developmental threshold necessary to metamorphose (Wilbur and Collins, 1973). In the 2014 experiment, we expected that fluctuations with an elevated mean temperature would have reduced mass at and time to metamorphosis

because the fluctuations reached temperatures that approached the thermal tolerance levels for the species (Manis and Claussen, 1986; Vasseur et al., 2014). Finally, we predicted that an increased magnitude in fluctuations would also cause reduced mass at metamorphosis and time to metamorphosis because of exposure to temperatures close to the species' critical thermal maximum (Manis and Claussen, 1986; Kern et al., 2015).

## MATERIALS AND METHODS

*Study Systems and Sites.*—*Lithobates sylvaticus* is a widespread terrestrial frog with explosive breeding in early spring, typically beginning in March in Connecticut (Moore, 1939; Conant and Collins, 1998). Wood frogs use small vernal pools for reproduction, egg deposition, and larval development (Moore, 1939). Tadpoles typically emerge from the vernal pools by late May (Moore, 1939). Upon metamorphosis, juveniles migrate into moist, terrestrial forest habitats (Conant and Collins, 1998).

In contrast, *H. versicolor* is a widespread, primarily arboreal frog with prolonged summer breeding, lasting from April to July in Connecticut (Conant and Collins, 1998; Klemens, 1993). *Hyla versicolor* frogs utilize vernal pools for reproduction, egg deposition, and larval development (Klemens, 1993; Conant and Collins, 1998). Juveniles generally emerge from these ephemeral pools by July, migrating to preferred arboreal habitats (Klemens, 1993; Conant and Collins, 1998). Ultimately, *L. sylvaticus* and *H. versicolor* were chosen because they are abundant in the northeast United States and because they are contrasting spring and summer breeding species with larvae that do not overwinter.

The collection site was an open canopy pond in the UConn Forest that is surrounded by a deciduous oak/hickory forest and contains elm/ash/red maple and red/white pine stands (Wharton et al., 2009; Parent and Volin, 2014). We collected three late-stage *L. sylvaticus* egg

masses collected from the pond on 20 April 2013 and 11 April 2014, and six amplexant *H. versicolor* adult frogs from the same pond on 21 May 2013, which then oviposited in containers. We released adults the next evening at the capture site. We brought all embryos to a cold room in the Aquatics Animal Care Facility at the University of Connecticut where they remained for the duration of the experiment. Embryos and emerging tadpoles were subjected to the same conditions until they were free swimming.

*Experimental Design.*— We completed three experiments to test the effects of the frequency and magnitude of temperature variability in the absence of interspecific competition, predation, and food limitation. We completed two experiments in 2013, one with *L. sylvaticus* and another with *H. versicolor*, using similar experimental temperature treatments. We completed a third experiment in 2014 with *L. sylvaticus* and a modified experimental design.

We tested five temperature treatments in 2013 that varied in the frequency and magnitude of fluctuation while maintaining the same mean temperature in all treatments. Treatment names reflect the change in water temperature with respect to ambient air temperature (Table 1). We maintained the control treatment (referred to as +3 constant), without fluctuation, at a temperature 3 °C above the ambient air temperature for the duration of the experiment. Heating the control tanks above the ambient air temperature allowed our fluctuating water temperature treatments to fluctuate above and below the control water temperature while maintaining the same mean temperature. The first fluctuating treatment (referred to as +3 weekly) fluctuated +/- 3 °C around the +3 control treatment every six days; the second treatment (referred to as +3 biweekly) fluctuated +/- 3 °C around the +3 control treatment every 12 days. We choose the six-day frequency because, on average, weather fronts in New England pass every 5 – 6 days, and this frequency accommodated a three-day water-change cycle. We then halved this frequency for

comparison. We also wanted to test the effects of an ecologically extreme event such as a heat wave (Ganguly et al., 2009; Gao et al., 2012), and thus we created two treatments with a single, large-magnitude temperature fluctuation that occurred early or late in the larval development period. The +9 high-early treatment entailed a 6 °C increase in temperature relative to the +3 constant for six days at the beginning of the experiment. The +9 high-late treatment entailed a 6 °C increase in temperature for six days at the end of the experiment as tadpoles approached metamorphosis.

In New England, mean daily air temperatures change greatly throughout the spring; therefore, the 2013 experiment with *L. sylvaticus* started with an ambient room temperature of 15 °C and increased by 1 °C every six days such that air temperatures in the lab reflected seasonal temperature increases outside. We changed the ambient air temperature on the days in which the treatment temperatures were readjusted and accordingly increased all tank temperatures by 1 °C to offset the increase in ambient air temperature. The maximum cold room temperature was 20 °C. Upon reaching this temperature on 30 May, we held the ambient air temperature constant at 20 °C and no longer subjected the tanks to an additional 1 °C temperature increase. The cold room was at the maximum room temperature when the *H. versicolor* experiment began, and thus we held the ambient air temperature at 20 °C for the duration of that experiment.

We tested an additional five temperature treatments in 2014 on *L. sylvaticus* that varied in the mean and magnitude of fluctuation. We used two constant temperature treatments where tanks were held at 2 and 6 °C above the ambient air temperature (referred to as +2 constant and +6 constant, respectively). We created two fluctuating treatments that varied +/- 2 °C around the +2 constant and +6 constant treatments (referred to as +2 weekly and +6 weekly, respectively). The last treatment (referred to as +4 weekly) fluctuated between the low and high temperatures

experienced by tadpoles in the previous two fluctuating treatments, such that these tanks experienced a  $\pm 4$  °C fluctuation from 20 to 28 °C and a mean of 4 °C above the ambient air temperature. As in the 2013 experiment, the fluctuating temperature treatments varied every six days to reflect average weather patterns and to accommodate a three-day water change cycle. We held the ambient air temperature in the cold room at a constant 20 °C for the duration of the 2014 experiment.

Experiments were performed between 22 April 2013 and 17 July 2013 and between 17 April 2014 and 3 June 2014. For each experiment, ten (2013) and 8 (2014) free-swimming tadpoles were haphazardly assigned to plastic cups and then randomly assigned to 19-liter aquarium tanks filled with 17 liters of reconstituted RO water. RO water was reconstituted with a 10% Holtzfreter solution for the 2013 *L. sylvaticus* experiment. The *L. sylvaticus* experiment initially began on 22 April, but errors in reconstituting RO water resulted in mortality, so we restarted the experiment on 30 April with surplus tadpoles from the initial collection. The *H. versicolor* experiment began on May 30. Due to the presence of tail kinks in our tadpoles, we used RO Rite (Kent Marine®), which contains additional trace minerals, to reconstitute the water for the *H. versicolor* experiment in 2013 and in the 2014 experiment. Conductivity was similar for both methods and reflected levels found in the natal pond. Treatments were randomly assigned to tanks. Tanks were randomly assigned without replacement to one of five spatial blocks such that each experimental block contained one tank from each treatment. The location of spatial blocks within the cold room and the location of tanks within each spatial block were randomly reassigned every six days to further minimize effects of temperature and light gradients within the room. To heat the tanks, we suctioned a single 50W Hydor Aquarium Heater to the top corner of each tank. Partial water changes were conducted every three days. These

water changes unavoidably resulted in temperature fluctuation within tanks that lasted less than 3 hours. To minimize this disturbance, we replaced heaters as quickly as possible. All tanks were fed *ad libitum* a combination of 50% rabbit chow and 50% tetramin.

Animals were removed from the aquariums when at least one front leg erupted (stage 42; Gosner 1960). During tail resorption, metamorphosed individuals were housed in plastic containers with a small amount of water to protect against desiccation and positioned to allow for the movements in and out of water. Once the tail was fully absorbed, we recorded the date of metamorphosis and mass.

*Data Analysis.*— We averaged mass and age at metamorphosis data for each tank and used linear models to estimate effects of spatial block and temperature treatments. Each model initially included survival (i.e., number of individuals that metamorphosed from a tank) to account for density-dependent effects resulting from mortality. Survival was removed from models when not significant. Diagnostic plots were used to assess whether the data met model assumptions. Data for any tanks identified as outliers were rechecked, but there was no reason to exclude these tanks from analysis. Finally, we used Tukey-adjusted *post hoc* tests to make pairwise comparisons between treatments. All data analyses were carried out in R version 3.0.2 (R Development Core Team 2013).

## RESULTS

*2013.*— Survival (i.e., number of individuals that metamorphosed from a tank) was significantly related to mass at metamorphosis of *L. sylvaticus* ( $F_{1,15} = 33.79$ ,  $p < 0.001$ ) and *H. versicolor* ( $F_{1,15} = 16.20$ ,  $p = 0.001$ ). High survival resulted in low mass at metamorphosis for both species. Survival also affected time to metamorphosis for *L. sylvaticus* ( $F_{1,15} = 10.26$ ,  $p = 0.006$ ) but not for *H. versicolor* ( $F_{1,15} = 2.58$ ,  $p = 0.129$ ). Wood frogs in tanks with high survival



took longer to reach metamorphosis than wood frogs in tanks with low survival. Temperature treatments in the wood frog experiment did not significantly relate to mass at ( $F_{4,15} = 1.52$ ,  $p = 0.247$ ) or time to metamorphosis ( $F_{4,15} = 0.40$ ,  $p = 0.807$ ) (Figure 1a. and 1c.). In contrast, gray tree frogs responded to temperature treatments as measured by both mass at ( $F_{4,15} = 5.05$ ,  $p = 0.009$ ) and time to metamorphosis ( $F_{4,16} = 4.35$ ,  $p = 0.014$ ) (Figure 1b. and 1d.). Tukey HSD post hoc tests revealed that gray tree frog tadpoles from +9 high-late were smaller than tadpoles from the +3 weekly ( $p = 0.013$ ) and +3 biweekly ( $p = 0.017$ ) fluctuation treatments. Thus, tadpoles exposed to a single, high magnitude spike in temperature late in development metamorphosed significantly smaller than tadpoles exposed to consistent weekly or biweekly temperature fluctuations. Tadpoles from the +3 biweekly fluctuation treatment took longer to reach metamorphosis than both the +9 high-early ( $p = 0.019$ ) and +9 high-late fluctuation treatments ( $p = 0.029$ ). In other words, tadpoles exposed to consistent biweekly temperature fluctuations developed more slowly than either of the single, high magnitude temperature treatments. All treatments finished with similar final average temperatures (Table 2).

2014.—Survival did not significantly relate to mass ( $F_{1,15} = 0.61$ ,  $p = 0.449$ ) or time to metamorphosis ( $F_{1,15} = 0.88$ ,  $p = 0.363$ ) of *L. sylvaticus* and thus was removed from the analyses. Temperature treatments influenced time to metamorphosis ( $F_{4,16} = 11.77$ ,  $p < 0.001$ ) but not mass at metamorphosis ( $F_{4,16} = 0.93$ ,  $p = 0.474$ ) (Figure 2a. and 2b.). Tadpoles from the +6 weekly and +6 constant treatments reached metamorphosis significantly earlier than tadpoles from the +2 constant, +2 weekly, or +4 weekly temperature treatments.

## DISCUSSION

As anticipated, *H. versicolor* performance was affected by fluctuating temperature treatments. *Hyla versicolor* tadpoles exposed to an extreme temperature fluctuation late in

development had a smaller mass and earlier emergence relative to the consistent weekly and biweekly temperature fluctuation treatments. Unexpectedly, *H. versicolor* developing under a biweekly temperature fluctuation pattern had later emergence compared to the two high magnitude temperature treatments. Also contrary to our predictions, performance responses to repeatedly fluctuating treatments did not differ significantly from constant temperatures held at the same mean for *L. sylvaticus* in either experiment. However, elevated mean temperatures of 26 °C in the 2014 experiment reduced time to metamorphosis, which was expected.

While a number of studies have examined the impact of daily thermal fluctuations (Niehaus et al., 2006; Niehaus et al., 2011; Arrighi et al., 2013; Rojas et al., 2014; Kern et al., 2015), our study focused on fluctuation frequencies pertaining to changing weather fronts and heat waves. We did not find reduced mass at metamorphosis or reduced development time in our fluctuating treatments relative to our controls. We only found such a response by *H. versicolor* in the +9 high-late treatment where such a response is likely to benefit tadpole fitness (Wilbur and Collins, 1973; Atkinson, 1996; Laurila and Kujasalo, 1999). Thus, we suggest that the reduced larval performance found by Niehaus et al. (2006) with daily temperature fluctuations was due to compounding effects and/or greater physiological stress than our less frequent temperature fluctuations. In support of this, it has been shown that mosquitoes exposed to daily temperature fluctuations have altered thermal sensitivities with lower critical maximum temperatures than mosquitoes exposed to constant temperatures (Paaijmans et al., 2013). This made the mosquitoes from the fluctuating treatments more susceptible to the increased temperatures predicted under climate change (Paaijmans et al., 2013). Another study examining the effects of daily temperature fluctuations found that development time of tadpoles from highly thermally variable environments was reduced compared to tadpoles from less thermally variable habitats (Kern et

al., 2015). However, in this same study, all species exposed to the daily temperature fluctuations had reduced growth relative to the control (Kern et al., 2015), which contradicts our findings.

The extreme fluctuating treatments from the 2013 *H. versicolor* experiment provide insight into the effect of high-magnitude, short-term temperature fluctuations at different points in development. As predicted, the extreme temperature fluctuation at the end of the larval period was enough of a temperature spike to induce early metamorphosis at a decreased mass compared to the consistent weekly and biweekly temperature fluctuation treatments. An extreme temperature spike early in larval development, however, was indistinguishable from the other treatments. Individuals exposed to the +9 high-early treatment likely had not yet crossed the physiological thresholds or the body size necessary to undergo metamorphosis at the time of the temperature fluctuation (Wilbur and Collins, 1973). Despite these thresholds, metamorphosis is a highly plastic process that is often delayed or accelerated to reflect resource availability, competition, predation, and environmental factors such as temperature (Wilbur and Collins, 1973). Early emergence can be an evolutionarily beneficial response to spikes in vernal pool temperatures as they dry (Atkinson, 1996). Under such a scenario, it is often beneficial to metamorphose, even at the cost of growth, than to risk increased competition, desiccation, and death (Wilbur and Collins, 1973; Atkinson, 1996; Laurila and Kujasalo, 1999). In comparison, we found that a consistent weekly or biweekly fluctuating pattern, albeit a smaller magnitude fluctuation, during the same time in development had the opposite effect as the +9 high-late treatment with late emergence for tadpoles exposed to the +3 biweekly treatment and increased mass at metamorphosis for tadpoles from both the +3 biweekly and +3 weekly treatments. This finding is consistent with observations that tadpoles benefit from remaining in stable aquatic environments to continue growth but metamorphosing under variable environments (Wilbur and

Collins, 1973). We suggest that the weekly and biweekly fluctuating temperature conditions were stable enough to delay metamorphosis and increase growth in contrast to a single, late-development extreme fluctuation.

We also note that the increased growth relative to the +9 high-late treatment and longer time to metamorphosis relative to the +9 high-late and +9 high-early responses exhibited by the *H. versicolor* tadpoles in the +3 biweekly treatment may be due to the prolonged exposure to depressed temperatures relative to the other treatments. We suggest that development rate was reduced during these times, but, since food was not limited in the laboratory, growth continued because growth and development are not coupled in tadpoles (Wilbur and Collins, 1973; Newman, 1998). *Hyla versicolor* tadpoles within control treatments with constant temperatures would not have experienced periods with slowed development but continued growth.

We also found evidence of species-specific responses to temperature variability such that *H. versicolor* responded to temperature variability more than *L. sylvaticus*. This may be due to differing adaptations to thermal variability or varied thermal optimum or tolerances (Kern et al., 2014). As a spring breeding species, *L. sylvaticus* may be exposed to more seasonal temperature variability than *H. versicolor* such that it is better adapted and responds less to temperature variability (Kern et al., 2015). This could explain why *H. versicolor* responded to the weekly, biweekly, +9 high-early, and +9 high-late treatments. However, it is also worth noting the thermal optimum and thermal tolerances of the species. *Lithobates sylvaticus* tadpoles from New York exhibit heat tolerances between 26 °C and 34 °C, which means that we reached or surpassed this thermal tolerance (Manis and Claussen, 1986). In order to increase larval survival, tadpoles at the upper reaches of their thermal tolerance should metamorphose (Wilbur and Collins, 1973), which is what we observed for *L. sylvaticus* tadpole exposed to a mean of 26 °C

throughout larval development. In comparison, the critical thermal maximum for *H. versicolor* tadpoles is approximately 42 °C, and we did not achieve temperatures on the higher end of the thermal performance curve (Katzenberger et al., 2014). In a thermally consistent environment within the range of thermally optimal temperatures, tadpoles are expected to increase development time (Wilbur and Collins, 1973, Katzenberger et al., 2014; Vasseur et al., 2014). The increased time to metamorphosis under the biweekly temperature fluctuation treatment relative to both high magnitude fluctuation treatments observed in our study could be explained in part by the fact that the temperature ranges our consistently fluctuating treatments approached the thermal optimum of 31 °C for *H. versicolor* tadpoles such that increasing development time could have benefited tadpole fitness (Katzenberger et al., 2014; Vasseur et al., 2014).

We found support for a potential developmental threshold of 26 °C for *L. sylvaticus* in our 2014 experiment relative to our other treatments based on the reduced time to metamorphosis observed in our +6 weekly and +6 constant treatments. While we expected earliest emergence from the +6 treatments, we anticipated that the +4 weekly treatment would have an emergence date that was intermediate between the +6 and +2 treatments because tadpoles in this treatment were exposed to an intermediate mean temperature of 24 °C and experienced higher magnitude fluctuations ( $\pm 4$  °C) than the +6 and +2 treatments. This pattern was not observed and may suggest a thermal threshold of 26 °C for *L. sylvaticus* in our experiment. *Lithobates sylvaticus* tadpoles from New York acclimated to 15 °C and 20 °C exhibited heat tolerances of about 26 °C and 34 °C, respectively (Manis and Claussen, 1986). Therefore, by reaching mean temperatures of 26 °C for the duration of development, we approached previously established thermal tolerances for *L. sylvaticus* tadpoles which could have caused earlier metamorphosis in our elevated treatments compared to our other treatments (Wilbur and Collins, 1973; Manis and

Claussen, 1986). Additionally, such a threshold for developmental suppression has been supported by a number of studies on invertebrates and reptiles (Hagstrum and Milliken, 1991; Georges et al., 2005; An et al., 2009). We note, however, that there was no difference between the +6 weekly and +6 constant treatments, despite the fact that the +6 weekly treatment surpassed the previously recognized 26 °C heat tolerance threshold by 2 °C every other week (Manis and Claussen, 1986). However, if 26 °C is a tipping point on the *L. sylvaticus* tadpole thermal performance curve, adverse affects from variable temperatures relative to the control may not yet be apparent at this temperature (Vasseur et al. 2014).

We did not detect differences in time to or mass at metamorphosis in the 2013 *L. sylvaticus* experiment; however, we acknowledge a high number of mortalities early in the experiment caused significant differences in tank densities (some tanks had as few as 5 tadpoles while others had the full 10). Additionally, because we had to restart this experiment with surplus tadpoles, there were small size disparities among the stocked tadpoles that likely exacerbated size differences among the tadpoles as the experiment progressed (Peacor and Pfister, 2006). Tail kinks also became apparent just before metamorphosis in about 32.6% of the surviving tadpoles. We suspect that the tail kinks developed because our water reconstitution method did not contain trace minerals necessary for development. We began feeding 80% rabbit chow and 20% tetraamin to prevent further tail kinks. Ultimately, tank densities (i.e., survival) significantly affected time to and size at metamorphosis for *L. sylvaticus*, emphasizing the importance of density-dependent and water-related stressors to *L. sylvaticus* performance and possibly overshadowing potential treatment effects.

Due to equipment and facility limitations, temperatures for the present experiments ranged from 15 to 29 °C and were unable to surpass the critical thermal temperatures for either

species (Manis and Claussen, 1986; Katzenberger et al., 2014). Despite this, we observed significant performance responses to temperature variability with unlimited food and in absence of competition, predators, and any other environmental factors. Thus our results are informative to research that tests the effects of multiple stressors and interactions with temperature on the performance of anurans (Van Meter et al. 2011; Li et al., 2013; Rohr and Palmer, 2013; Rumschlag et al., 2014). We found that *L. sylvaticus* reduced development time at mean temperatures of 26 °C and that *H. versicolor* exhibited a reduced mass at metamorphosis relative to consistent weekly and biweekly temperature fluctuations when exposed to high magnitude fluctuations at the end of development. *H. versicolor* also exhibited increased time to metamorphosis under the biweekly temperature fluctuation treatment relative to both high magnitude fluctuation treatments. Additional testing that is able to reach and surpass the critical thermal maximum for *L. sylvaticus* and *H. versicolor* should have the potential to produce a more complete gradient of responses to thermal variability. In accordance with the thermal performance curves of our species, our results show species-specific responses to thermal variability such that adaptation to thermal variability and thermal optimum and tolerances may vary (Manis and Claussen, 1986; Katzenberger et al., 2014; Kern et al., 2015). Amphibian declines worldwide are likely due to a combination of climate change, pollution, disease, habitat alteration, and overexploitation (Kiesecker et al., 2001; Collins and Storfer, 2003; Li et al., 2013; Rohr and Palmer, 2013), thus it is imperative that we try to gain a better understanding of how climate change and its interactions will affect amphibian performance and survival (Li et al., 2013). Additional research that incorporates temperature variability, especially in the context of climate change, is strongly advised to more accurately represent natural temperature regimes and

the effects current and future climates have on the performance and fitness of amphibian populations.

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**Table 1.** A list of the treatment names, the magnitude of their fluctuations, and the frequency of their fluctuations for the 2013 and 2014 experiments. We note that the mean temperature of the 2013 *L. sylvaticus* experiment increased incrementally from 18 °C to the 23 °C. The final 23 °C water temperature is reflected below.

<b>Temperature Treatments 2013</b>			
<b>Treatment Name With Respect to Ambient Air Temperature</b>	<b>Mean Treatment Temperature</b>	<b>Fluctuation Magnitude With Respect to the Treatment Mean</b>	<b>Fluctuation Frequency</b>
+3 constant	23	0	0
+3 weekly	23	+/- 3	every 6 days
+3 biweekly	23	+/- 3	every 12 days
+9 high-early	23	+6	once for 6 days
+9 high-late	23	+6	once for 6 days

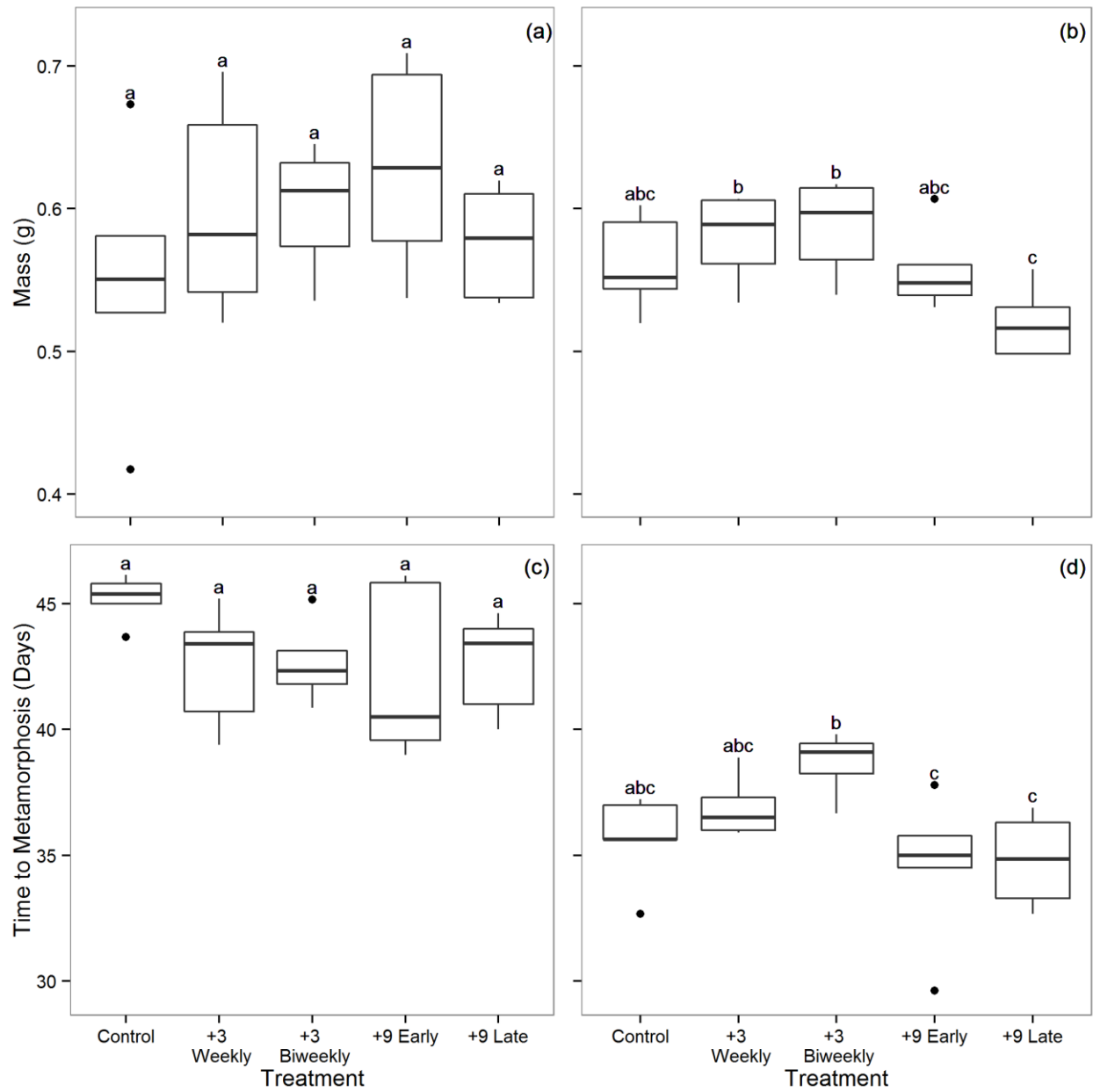
<b>Temperature Treatments 2014</b>			
<b>Treatment Name With Respect to Ambient Air Temperature</b>	<b>Mean Treatment Temperature</b>	<b>Fluctuation Magnitude With Respect to the Treatment Mean</b>	<b>Fluctuation Frequency</b>
+2 constant	22	0	0
+6 constant	26	0	0
+2 weekly	22	+/- 2	every 6 days
+4 weekly	24	+/- 4	every 6 days
+6 weekly	26	+/- 2	every 6 days



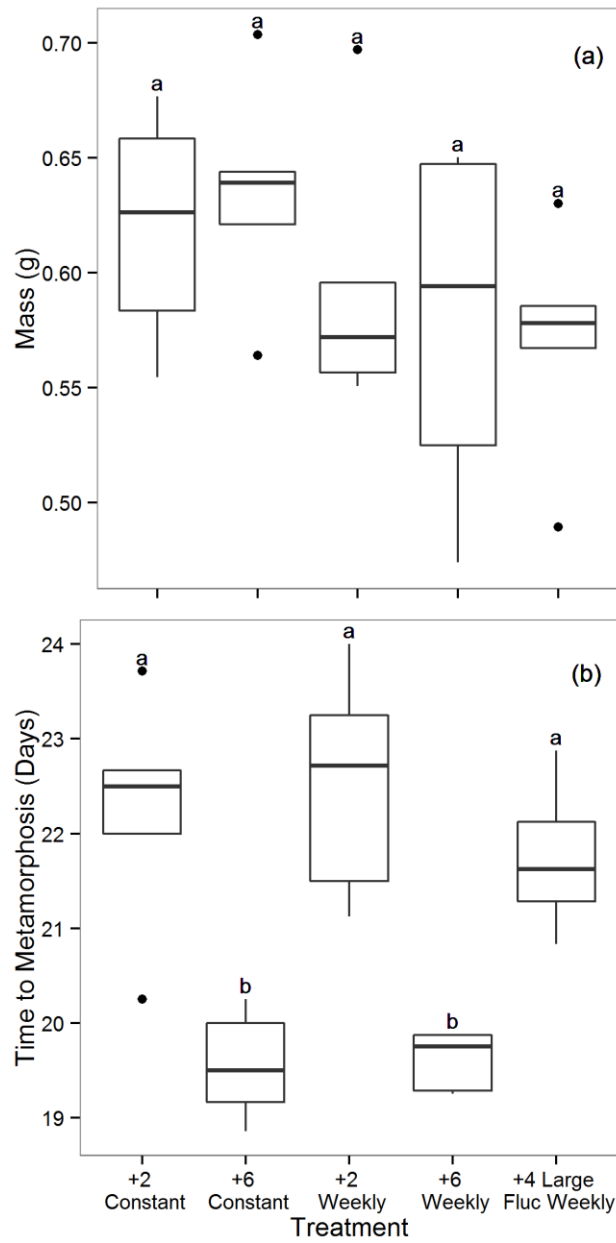
**Table 2.** The average temperatures and the average standard deviation for the 2013 *L. sylvaticus* and *H. versicolor* experimental tanks. The standard deviation for the averaged experimental tank temperatures is also included.

TreatmentTemperatureMeans2013Lithobates sylvaticus			
Treatment	Average Temperature (°C)	Average SD	SD of Averaged Temperatures
+3 Constant	21.95	1.61	0.36
+3 Weekly	22.19	2.94	0.39
+3 Biweekly	21.83	3.06	0.37
+9 High-Early	22.56	1.92	0.43
+9 High-Late	22.32	2.47	0.27

TreatmentTemperatureMeans2013Hyla versicolor			
Treatment	Average Temperature (°C)	Average SD	SD of Averaged Temperatures
+3 Constant	23.04	1.14	0.05
+3 Weekly	23.64	2.61	0.50
+3 Biweekly	24.14	2.63	0.38
+9 High-Early	23.74	1.61	0.56
+9 High-Late	23.70	1.82	0.31



**Figure 1.** Mass at metamorphosis for *L. sylvaticus* (a) and *H. versicolor* (b) and time to metamorphosis for *L. sylvaticus* (c) and *H. versicolor* (d) for each treatment in the 2013 experiment.



**Figure 2.** Mass at metamorphosis for *L. sylvaticus* (a) and time to metamorphosis for *L. sylvaticus* (b) for each treatment in the 2014 experiment.

Road salts, elevated temperatures, and competitive interactions impact wood frog (*Lithobates sylvaticus*) and spring peeper (*Pseudacris crucifer*) tadpole performance

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ABSTRACT.—Amphibian populations are declining throughout the United States as a result of local environmental stressors. Thus, we chose to examine how two local environmental stressors, road salt deposition and elevated average seasonal temperatures, would impact wood frog (*Lithobates sylvaticus*; LISY) and spring peeper (*Pseudacris crucifer*; PSCR) tadpole performance. Road salts have already been shown to reduce larval anuran survival and increase mass at metamorphosis as a result of altered trophic interactions while elevated seasonal temperatures result in early emergence of metamorphs from vernal pools. We were interested in the potential synergistic interactions between road salt, elevated temperature, and interspecific competition. Thus, we performed a fully randomized and replicated mesocosm experiment in which all possible combinations of competition (no or yes), temperature (ambient or elevated 3 °C), and salt (ambient or 1600 mg/L chloride) were tested. As anticipated, road salts reduced PSCR survival and increased mass. However, we observed an interaction between road salt and competition such that PSCR survival and mass at metamorphosis increased under such scenarios, suggesting that additional periphyton growth in the salt treated tanks reduced the resource limitation induced by the competition treatments. We found support for multiple stressors independently affecting LISY survival and mass at metamorphosis. Both elevated road salt and elevated temperature individually reduced LISY survival. Likewise, elevated road salt and elevated temperature separately increased LISY mass at metamorphosis. As predicted, both species emerged earlier under elevated temperature treatments. LISY and PSCR also exhibited increased development time under interacting salt and temperature treatments, perhaps as a result of altered osmoregulatory processes slowing development. We urge the consideration of multiple local stressors and potential synergistic interactions between local environmental stressors when managing amphibian populations in the future.

*Key Words:* Climate change; road salt; amphibian; interaction; conservation

## INTRODUCTION

Empirical data from monitoring of local amphibian populations has revealed a consistent decline rate of 3.79% per year throughout the United States (Grant et al., In Press). Furthermore, the changing global climate poses a new threat to amphibian populations that may interact synergistically with other environmental stressors to adversely affect amphibian populations (Urban, 2015). Given the complexity of amphibian population declines, there is a growing need to identify and understand the multiple stressors affecting local amphibian populations and the potential synergistic interactions among stressors (Rohr and Palmer, 2013; Li et al., 2013; Dalinsky et al., 2014; Rumschlag et al., 2014). Here we will focus on two local stressors to amphibian populations: elevated seasonal temperatures due to climate change and the infiltration of road salts into wetland ecosystems.

Predictive climate change models for the northeastern part of the United States show increases in average annual temperatures by as much as 3 °C by 2050 (Hayhoe et al., 2008) and seasonally dependent changes to temperature and precipitation patterns that could lead to water deficits in the summer (Hayhoe et al., 2008; Brooks, 2009). Such climatic shifts could cause vernal pools to dry early and stay dry for longer periods of time, adversely affecting amphibian populations through reduced recruitment success and altered community interactions and assemblages (Atkinson, 1996; Brooks, 2004; Hayhoe et al., 2008; Brooks, 2009; Salice, 2012; Rohr and Palmer, 2013). Additionally, the changing global climate has already altered the phenology of breeding and spring emergence for amphibians, with many species initiating these behaviors much earlier than just a few decades ago (Gibbs and Breisch, 2001; Todd et al., 2011;

Klaus and Loughheed, 2013; Benard, 2015). Ultimately, phenological shifts can lead to resource mismatch and altered wetland community interactions (Todd et al. 2011). Climate change may also cause distributional shifts in amphibian populations that could create mismatched communities, collapsed distributions, and remnant populations that are genetically isolated and vulnerable to stochastic events (Parmesan and Yohe 2003; Hickling et al. 2006; Holsinger 2012). Climate also has the potential to interact with other environmental threats such as invasive species, disease, and pollutant exposure to have cascading affects on amphibian populations and their ecosystems (Rohr et al. 2010; Kattwinkel et al. 2011; Bellard et al. 2013; Li et al. 2013; Saenz et al. 2013; Rohr and Palmer, 2013; Rumschlag et al., 2014). For example, moderate levels of climate change combined with stressors such as herbicide exposure could alter behavior, reduce mass, and increase mortality in larval amphibians (Rohr and Palmer, 2013).

The deposition of road salts during the winter to de-ice roads for human safety is a practice that has increased substantially over the last few decades with no foreseeable reduction in use (Novotny, 2010). Road salt is a common environmental stressor throughout New England with documented negative effects on amphibians which increase as distance to a roadside decreases (Karraker et al., 2008; Karraker and Gibbs, 2011; Hopkins et al., 2013; Dalinsky et al., 2014; Dananay et al., 2015). High salt concentrations have the potential to influence every amphibian lifestage and have been linked to reduced amphibian densities, developmental deformities, disruption of osmoregulatory processes, reduced fitness, and altered amphibian behavior (Karraker et al., 2008; Denoël et al., 2010; Karraker and Gibbs, 2011; Dananay et al., 2015). Although salts flush from stream systems, strong legacy effects of road salts can persist in pond and wetland systems and alter productivity and trophic interactions in these systems (Novotny, 2009; Van Meter et al. 2011; Dalinsky et al., 2014). Van Meter et al. (2011) found

that road salt additions in a mesocosm experiment reduced zooplankton densities such that the standing stock of periphyton and phytoplankton increased. As a result of this increased resource availability, tadpoles emerging from salt treated tanks had an increased mass at metamorphosis (Van Meter et al., 2011). However, when examined under conditions without resource limitation, salt has no effect on mass (Petranka and Francis, 2013). Additionally, wood frog tadpoles raised under chronic, high salinity conditions in isolation of other environmental factors showed no significant difference in mass compared to control tadpoles raised under normal freshwater conditions (Sanzo and Hecnar, 2006). Despite the potential benefit to growth in natural systems, another study found negative legacy effects in juvenile frogs that were exposed to road salts during larval development (Dananay et al. 2015). Ultimately, the altered trophic interactions caused by road salts could have cascading effects on natural wetland community interactions (Wilbur, 1997; Van Meter et al., 2011).

Tadpole performance responses have been quantified under road salts (Karraker et al., 2008; Van Meter et al., 2011; Dananay et al., 2015), elevated temperature scenarios (Bustamante et al., 2010; Rohr and Palmer, 2013), and competition (DeBenedictis, 1974; Skelly, 1995b; Peacor and Pfister, 2006); however, there is a need for further study of the interactions between these factors because multiple, local stressors are often the cause of amphibian declines and many studies warn of the interactive effects of elevated temperatures (Grant et al., In Press; Saenz et al. 2013; Rohr and Palmer, 2013; Rumschlag et al., 2014). Thus, we examined the interactions between road salt, elevated temperature, and competition between tadpole species by completing a fully factorial mesocosm experiment in which all possible combinations of competition (no or yes), temperature (ambient or elevated), and salt (ambient or elevated) were tested. We predicted that both species would have similar responses to the factors of interest. We



predicted that road salt would reduce tadpole survival (Karraker et al., 2008) and increase mass at metamorphosis (Van Meter et al. 2011), while elevated temperature would result in early emergence (Atkinson, 1996). We also expected both species to be fairly robust to the low levels of competition in the absence of other stressors (DeBenedictis, 1974; Skelly, 1995a; Skelly, 1995b; Smith and Van Buskirk, 1995; Peacor and Pfister, 2006). However, we were primarily interested in the interactions between the main effects. We predicted that elevated temperatures would exacerbate the effect of salt due to increased thermal stress based on studies finding adverse interactive effects with elevated temperatures (Rohr and Palmer 2013; Rumschlag et al. 2014). We anticipated that elevated salt would produce tadpoles with a greater mass due to increased periphyton availability and that competition would interact synergistically such that mass at metamorphosis would be affected (DeBenedictis, 1974; Skelly, 1995b; Van Meter et al., 2011; Baldwin et al. 2014; Dananay et al., 2015). Finally, we predicted that exposure to elevated temperature treatments would result in early emergence (Klaus and Loughheed, 2013) with competition also contributing to early emergence (DeBenedictis, 1974; Skelly, 1995b).

## MATERIALS AND METHODS

*Study Sites and Systems.*— *Lithobates (Rana) sylvaticus* (LISY; LeConte, 1825; Frost et al., 2006) is a terrestrial frog with a wide geographic range and a complex life cycle (Berven, 1990). *Lithobates sylvaticus* use small vernal pools for explosive early spring reproduction, egg deposition, and the larval life stage (Berven, 1990). In Connecticut, *L. sylvaticus* tadpoles occupy vernal pools from March until late May during which time they are generalist foragers that can obtain nutrients from substrates that may be purposely or inadvertently eaten and can resort to cannibalism during times of limited resource availability (Seale and Beckvar, 1980; Altig et al., 2007; Schiesari et al., 2009; Jefferson et al. 2014). Juveniles will then metamorphose starting

in late May and continuing until early July, at which point the juveniles migrate into moist, terrestrial forest habitats for the summer.

*Pseudacris crucifer* (PSCR; Weid-Neuweid, 1838) is a semi-arboreal frog with a wide geographic range covering all of the United States east of the Mississippi and as far north as Hudson Bay (Lovett, 2013). Although widespread, this species is restricted to woodland areas, where adults breed in open canopy ponds early in the season, generally between March and April in northern populations (Lovett, 2013). Breeding typically occurs in temporary or semi-permanent open-canopy wetlands (Conant and Collins, 1998; Lovett, 2013). Larval development continues until early July with tadpoles often co-occurring with *L. sylvaticus* tadpoles and other larval amphibians (Skelly, 1996; Conant and Collins, 1998). In contrast to *L. sylvaticus*, *P. crucifer* forage primarily on periphyton as larva (Skelly, 1995a).

The collection site was an open canopy pond in the University of Connecticut Forest, which is surrounded by a deciduous oak/hickory forest that also contains elm/ash/red maple and red/white pine stands (Wharton et al., 2009; Parent and Volin, 2014). We collected five late-stage *L. sylvaticus* egg masses from the pond on 12 April 2014 and five amplexant *P. crucifer* adult frogs from the same pond on 13 April 2014 in order to collect eggs. We released the frogs the following evening at the capture site. We brought eggs to an animal care facility at the University of Connecticut, Storrs, CT, where the eggs hatched and were held until they reached the free-swimming stage. We stocked the tadpoles into one of 48 mesocosm tanks on 27 April 2014, where they were subjected to one of 12 experimental treatments until the final tadpole reached metamorphosis on 4 July 2014.

*Experimental Design.*— In a fully randomized and replicated experiment, we randomly assigned one of 12 treatments to each 1000-liter mesocosm (N = 48 mesocosms) such that tanks

had all possible combinations of species (LISY, PSCR, and BOTH), temperature (ambient or elevated), and salt (ambient or elevated). Tanks were also organized into four spatial blocks to account for potential environmental gradients at our study site.

Each mesocosm represented a natural wetland. Thus, we filled mesocosms with ground water, which we allowed to age for two days. To each mesocosm, we added 1 kg of leaf litter collected from a mixed hardwood forest located within the University of Connecticut's Fenton Tract (Wharton et al., 2009; Parent and Volin, 2014). We covered mesocosms with 50% shade cloth lids to represent canopy cover and to prevent other amphibian species and dragonflies from ovipositing in the water. We then inoculated each mesocosm with a concentrated mixture of phyto- and zooplankton collected from multiple natural ponds on 17 April. Zooplankton do not serve as a food source for tadpoles, but are important components of the complex communities found in natural ponds (Schell et al., 2001).

We created the elevated salt treatment by adding road salt obtained from the Department of Transportation storage facility in Mansfield, CT. We dissolved road salt into 15 L of water and then stirred salt water into the mesocosms until we reached a concentration of 1600 mg/L on 21 April. We created the elevated by 3 °C temperature treatment using JBJ True Temp Heating Systems (Model T3-1000) which consists of a 1000 watt titanium heating rod and digital controller. We floated the heated rod 10 cm below the surface of the water, such that a natural temperature gradient occurred with the warmest water at the surface and coolest water at the bottom. The digital controller provided a set point temperature, but could not be programmed to follow daily temperature fluctuations, and the thermometer linked to the controller was placed at the bottom of the tank. We adjusted temperature set points in the morning, by programming the set-point temperature to be 3 °C warmer than the current morning temperature at the bottom of

control tanks. Set-point temperatures were not changed daily, but rather every three to five days. Adjustments, both increasing and decreasing the set-point, were needed following weather fronts causing large shifts in daily high or low temperatures and when rain fell which added water of a different temperature to the mesocosms.

We stocked mesocosm tanks on 27 April with 30 tadpoles of LISY, PSCR, or BOTH species; mesocosms assigned to BOTH received 30 tadpoles of each species for a total of 60 tadpoles. This density is well within the range of larval anuran densities in natural wetlands (Morin, 1983). We removed animals when at least one front leg erupted (stage 42; Gosner 1960), and these individuals were identified as surviving the larval lifestage. We placed metamorphosed individuals into plastic sandwich containers with a small amount of water from the mesocosm to protect against desiccation during tail absorption and positioned the containers to allow for movement of metamorphs in and out of water. We labeled containers with the corresponding mesocosm tank number and held the animals in the adjacent animal care facility through tail resorption. We checked the containers daily to assess progress through metamorphosis. We recorded the date of metamorphosis and mass at the completion of resorption. We calculated all "days to metamorphosis" using the date of complete tail resorption, rather than capture date, because some metamorphs were captured at different stages in the tail resorption process.

Water temperature, specific conductivity, and dissolved oxygen (DO) were measured biweekly in all mesocosms using a handheld sonde (Yellow Springs Instruments, YSI 556 MPS). Water samples were collected biweekly and filtered immediately through 0.7um GF/F Whatman filters in syringe filter holders. Filtered samples were collected in acid washed and field rinsed bottles, transported to the lab on ice, and frozen until analysis. Filtered samples were analyzed according to standard methods (APHA, 1998) for soluble reactive phosphorus (SRP; ascorbic

acid method), ammonium ( $\text{NH}_4^+$ ; phenate method), and nitrate ( $\text{NO}_3^-$ ; cadmium reduction method) at the Center for Environmental Sciences and Engineering Nutrients Laboratory. Dissolved inorganic nitrogen (DIN) was calculated by summing  $\text{NH}_4^+$  and  $\text{NO}_3^-$  concentrations. Filters were preserved by freezing, and chlorophyll a concentrations were measured by acetone extraction on a Turner Designs fluorometer (Trilogy 7000-000).

*Data Analysis.*—All analyses were performed for LISY and PSCR separately to determine species-specific responses to the salt and temperature manipulations. We used the mesocosms with both species to examine the effect of competition on the focal species. We fit general linear mixed effects models (GLMM) that incorporated the three, two-level factors (competition, temperature, and salt) and all possible interactions between the factors. We completed the same analysis for the three response variables (survival, mass at metamorphosis, and time to metamorphosis). We included Block and Tank in the models as random intercepts such that we could account for tank-level variability and any potential variation due to environmental gradients at the study site.

We modeled survival, measured as 1 = survived to metamorphosis and 0 = death before metamorphosis, using a binomial distribution. We modeled mass at metamorphosis and time to metamorphosis using a normal Gaussian distribution and included survival as a covariate to account for density dependent effects on these two responses. For each GLMM, we performed an analysis of deviance using type II Wald chi-square tests. Finally, we used Tukey *post hoc* contrasts of least-squares means to make pairwise comparisons between the factors and interaction terms.

We analyzed the water chemistry data using general linear models (GLM). As before, we performed the analyses separately for each species to correspond to the analyses on tadpole

responses. The GLMs incorporated our two, two-level factors (temperature, and salt) and their interactions for each of the response variables (water temperature, conductivity, chlorophyll a, SRP, DO, and DIN). We used these GLMs to perform two-way ANOVA analyses and Tukey *post hoc* tests to make pairwise comparisons. We summarized data in Microsoft Excel and completed analyses using R version 3.1.3.

## RESULTS

*Summary Statistics.*— The first LISY metamorphs reached Gosner stage 42 on 25 March, and the first PSCR metamorphs reached Gosner stage 42 on 1 June (Gosner ,1960). The first metamorphs completely absorbed their tails on 29 May for LISY and 4 June for PSCR. Throughout the duration of the study the average time to complete metamorphosis was 2.92 days, with a range from 0 to 7 days for LISY. The average time to complete metamorphosis was 2.86 days, with a range from 1 to 13 days for PSCR.

We massed a total of 1092 metamorphs, 747 LISY and 345 PSCR. LISY had an average mass of 0.821 grams with a range from 0.379 to 2.9 grams. PSCR had an average mass of 0.241 grams with a range from 0.1 to 0.39 grams.

We had four mesocosms that failed to produce metamorphs: two from the treatment with PSCR, ambient temperature, and elevated salt, and two from the treatment with PSCR, elevated temperature, and elevated salt. Most tadpole deaths were never observed. We only encountered a total of 156 deceased metamorphs throughout the course of the study, 66 LISY, 43 PSCR, and 47 tadpoles in which the species was indistinguishable. These individuals were either found drowned in the mesocosms or did not survive tail absorption during metamorphosis. One mesocosm (both species, elevated temperature, elevated salt) was colonized by gray tree frogs

(*Hyla versicolor*; HYVE) and was removed from all statistical analyses. Heaters malfunctioned in two additional tanks and thus these tanks were removed from all statistical analyses.

*Effects on Tadpoles.*— LISY and PSCR survival was significantly influenced by our factors (Figure 1). The number of LISY tadpoles surviving to metamorphosis was reduced by both elevated road salt ( $p < 0.001$ ) and elevated temperature ( $p < 0.039$ ). Competition was not significant ( $p = 0.930$ ). The interaction term for salt and temperature was marginally significant ( $p = 0.067$ ) in increasing survival. The interaction term for competition and temperature was also marginally significant ( $p = 0.069$ ), decreasing survival. In the model for PSCR, the number of PSCR tadpoles surviving to metamorphosis was reduced by elevated salt ( $p < 0.001$ ). However, PSCR survival was not as severely reduced by competition in the presence of salt ( $p = 0.045$ ) (Figure 2). Neither competition ( $p = 0.149$ ) nor temperature ( $p = 0.073$ ) were significant. Overall, road salt additions decreased average LISY survival from 90.4% to 62.3% and average PSCR survival from 56% to 14%.

LISY and PSCR mass at metamorphosis was significantly impacted by our factors of interest (Figure 3). Elevated salt ( $p < 0.001$ ) and elevated temperature ( $p < 0.001$ ) increased mass at metamorphosis for LISY. An interaction between elevated salt and elevated temperature reduced mass at metamorphosis relative ( $p = 0.004$ ) (Figure 4). Reduced survival also increased mass at metamorphosis ( $p < 0.001$ ). Competition was not significant for LISY mass at metamorphosis ( $p = 0.794$ ). PSCR mass at metamorphosis was reduced by competition ( $p = 0.026$ ), but mass increased relative to the no competition treatment in the presence of salt ( $p = 0.016$ ) (Figure 5). Salt ( $p = 0.186$ ) and temperature ( $p = 0.492$ ) did not affect mass at metamorphosis. Reduced survival also increased mass at metamorphosis for PSCR ( $p < 0.001$ ).

LISY and PSCR days to metamorphosis were significantly related to our factors of interest (Figure 6). Elevated temperature reduced development time of LISY ( $p < 0.001$ ). An interaction between elevated salt and temperature was also observed with increased days to metamorphosis under the elevated temperature treatment in the presence of salt ( $p = 0.030$ ) (Figure 7). Salt ( $p = 0.529$ ) and competition ( $p = 0.336$ ) were not significant determinants of development time. Reduced survival also increased days to metamorphosis ( $p < 0.001$ ). Elevated temperature reduced development time of PSCR ( $p < 0.001$ ). An interaction between elevated salt and temperature was also observed with increased days to metamorphosis under the elevated temperature treatment in the presence of salt ( $p < 0.001$ ) (Figure 8). Salt ( $p = 0.198$ ) and competition ( $p = 0.835$ ) were not significant determinants of development time. Reduced PSCR survival also increased development time ( $p < 0.001$ ). Overall, elevated temperatures caused the median time to metamorphosis to decrease by 15.29 days for LISY and 13.89 days for PSCR.

*Effects of Water Chemistry.*—Our experimental manipulations effectively increased water temperature (Figure 9). Water temperature was higher in the LISY heated tanks ( $F_{1,26} = 76.48$ ,  $p < 0.001$ ). Likewise, water temperature was higher in PSCR elevated treatment tanks ( $F_{1,25} = 27.51$ ,  $p < 0.001$ ). Salt treatments had decreased water temperature for LISY ( $F_{1,26} = 7.14$ ,  $p = 0.013$ ). Salt treatments also had decreased water temperature for PSCR ( $F_{1,25} = 6.63$ ,  $p = 0.016$ ).

Our experimental manipulations also effectively increased specific conductivity (Figure 10). Conductivity was higher in LISY elevated salt treatments ( $F_{1,26} = 10163.51$ ,  $p < 0.001$ ) and salt and temperature interaction treatments ( $F_{1,26} = 99.49$ ,  $p < 0.001$ ), but lower in elevated temperature treatments ( $F_{1,26} = 95.02$ ,  $p < 0.001$ ). Conductivity was also higher in PSCR elevated salt treatments ( $F_{1,25} = 21281.9$ ,  $p < 0.001$ ) and salt and temperature interaction treatments ( $F_{1,25} = 219.3$ ,  $p < 0.001$ ), but lower in elevated temperature treatments ( $F_{1,25} = 253.2$ ,  $p < 0.001$ ).



Overall, the average specific conductivity for the no salt addition treatments was 147  $\mu\text{S}/\text{cm}$  (SD: 39  $\mu\text{S}/\text{cm}$ ) and 3500  $\mu\text{S}/\text{cm}$  (SD: 452  $\mu\text{S}/\text{cm}$ ).

We found elevated chlorophyll a in both LISY and PSCR salt treatments (Figure 11). Chlorophyll a was higher in LISY elevated salt treatments ( $F_{1,26} = 18.07$ ,  $p < 0.001$ ). Temperature treatment did not affect chlorophyll a ( $F_{1,26} = 0.003$ ,  $p < 0.957$ ). Chlorophyll a was also higher in PSCR elevated salt treatments ( $F_{1,25} = 27.80$ ;  $p < 0.001$ ) and for salt and temperature interaction treatments ( $F_{1,25} = 6.39$ ,  $p = 0.018$ ). Temperature treatment did not affect chlorophyll a in PSCR tanks ( $F_{1,25} = 0.29$ ,  $p = 0.594$ ).

We found increased soluble reactive phosphorus concentrations in both LISY and PSCR salt treatments (Figure 12). SRP concentration was higher in LISY elevated salt treatments ( $F_{1,26} = 21.34$ ;  $p < 0.001$ ). Temperature treatments did not affect SRP concentrations ( $F_{1,26} = 1.76$ ,  $p = 0.196$ ). SRP concentration was also higher in PSCR elevated salt treatments ( $F_{1,25} = 13.12$ ;  $p = 0.001$ ). Temperature treatments did not affect SRP concentrations ( $F_{1,25} = 2.04$ ,  $p = 0.166$ ). We note that trace minerals are often included in road salts; therefore, it is unclear whether the elevated SRP concentrations are caused by the altered chemical composition caused by adding road salts or if the SRP was introduced into the mesocosms as a trace mineral in the road salt.

We observed higher dissolved oxygen concentrations for both LISY and PSCR salt treatments (Figure 13). DO concentration was higher in LISY salt treatment tanks ( $F_{1,26} = 17.10$ ,  $p < 0.001$ ). Temperature treatment did not affect DO concentration ( $F_{1,26} = 2.66$ ,  $p = 0.115$ ). DO concentration was also higher in PSCR salt treatment tanks ( $F_{1,26} = 22.49$ ,  $p < 0.001$ ). Temperature treatment did not affect DO concentration ( $F_{1,26} = 3.77$ ,  $p = 0.064$ ).

We found no observable differences in the total dissolved inorganic nitrogen content for any of the LISY or PSCR tanks. DIN concentrations did not differ between salt ( $F_{1,26} = 2.98$ ,  $p =$

0.096) or temperature treatments for LISY ( $F_{1,26} = 0.23$ ,  $p = 0.632$ ). DIN concentrations also did not differ between salt ( $F_{1,25} = 1.39$ ,  $p = 0.249$ ) or temperature treatments for PSCR ( $F_{1,25} = 0.48$ ,  $p = 0.497$ ).

## DISCUSSION

Overall, we found that both species respond to the main effects of road salts, temperature, and competition and to synergistic interactions between the main effects. As expected, we observed reduced survival and increased mass at metamorphosis for both species under the elevated road salt treatments. However, we observed an interaction between road salt and competition such that spring peeper survival and mass at metamorphosis increased under such scenarios, suggesting that additional periphyton growth in the salt treated tanks reduced the resource limitation induced by the competition treatments. We also found support for multiple stressors independently affecting wood frog survival and mass at metamorphosis. Both elevated road salt and elevated temperature individually reduced wood frog survival. Similarly, elevated road salt and elevated temperature separately increased wood frog mass at metamorphosis. We also found that both species emerged early under elevated temperature treatments, which we anticipated. However, both LISY and PSCR also exhibited increased development time under interacting salt and temperature treatments, perhaps as a result of altered osmoregulatory processes slowing development.

Freshwater ecosystems often experience high pulses of road salt following precipitation or melting events in the spring and in urban environments (Sanzo and Hecnar, 2006; Novotny, 2010). We chose a road salt treatment (1600 mg/L  $\approx$  3045  $\mu$ S/cm) near the high end of conductivity values reported in the literature (2904.8  $\mu$ S/cm in New York by Karraker et al 2008). However, chloride concentrations as high as 4000 mg/L have been recorded in ponds and

wetlands in Canada (Sanzo and Hecnar, 2006). Our ambient salt treatments had an average of 147  $\mu\text{S}/\text{cm}$ , well within the range of specific conductivities observed in wetlands in northeast Connecticut (average = 71.3  $\mu\text{S}/\text{cm}$ , max = 358.6  $\mu\text{S}/\text{cm}$ ; personal communication with E. Hall). For both amphibian species, the addition of road salt to a concentration of 1600mg/L reduced survival. However, we note that due to drought conditions that summer, water level dropped in the tanks such that conductivity increased throughout the summer, increasing from an average of 3095  $\mu\text{S}/\text{cm}$  on 25 April to 3642  $\mu\text{S}/\text{cm}$  by 18 June. Previous research has shown that LISY survive well in low to moderate saline environments (Dananay et al. 2015), but the reduction in survival was higher than expected, especially for PSCR, which experienced complete mortality in four tanks. PSCR are typically robust to anthropogenic disturbances and are known to occupy altered habitats such as agricultural fields, cut forest stands, drainage ditches that most other anurans avoid (McLeod and Gates, 1998; Gibbs et al. 2005; Alix et al., 2014; Homyack et al., 2015). However, PSCR drop out of urban landscapes (Gibbs, 1998; Gibbs et al. 2005). We suggest that road salt infiltration into breeding wetlands as a possible mechanism explaining this pattern of low PSCR abundance and occupancy in urban landscapes (Gibbs et al. 2005; Gagné and Fahrig, 2010).

We were surprised to find that PSCR responded to competition with reduced mass and that an interaction between competition and road salt addition increased PSCR survival and mass at metamorphosis. The impact of competition was unexpected because PSCR tadpoles typically do not exhibit adverse performance responses to competition (Skelly, 1995a; Skelly, 1995b; Smith and Van Buskirk, 1995). However, the reduced PSCR mass at metamorphosis suggests exploitative and/or interference competition with LISY for limited periphyton resources. As a larger, successful generalist forager, LISY tadpoles may have been better equipped to exploit

foraging resources in our experimental tanks (Altig et al. 2007; Schiesari et al., 2009). Interestingly, mass at metamorphosis was greater under elevated salt and competition scenarios. As mentioned previously, increased salinity has the potential to indirectly increase larval amphibian mass through increased periphyton resource availability (Van Meter et al. 2011; Petranksa and Francis, 2013; Dalinsky et al., 2014; Dananay et al. 2015). The increased productivity under the salt treatments may have allowed PSCR tadpoles in the competitive treatment to better exploit available resources, ultimately increasing mass at metamorphosis. The elevated chlorophyll a, orthophosphate concentrations, and dissolved oxygen concentrations we observed in the elevated salt treatments further support an indirect effect of road salt increasing periphyton growth within our salt treated tanks. Density dependence in our competition tanks was not consistent with our non-competition tanks because they were stocked with 60 tadpoles (30 of each species) instead of 30 tadpoles (15 of each species). Thus, direct comparison between these treatments is not possible.

LISY survival and mass at metamorphosis appear to be predominantly affected by multiple independent stressors. In addition to elevated salt reducing survival, elevated temperature treatments also reduced LISY survival. This suggests that LISY tadpoles were thermally stressed at the temperature range of this experiment. Our elevated temperature treatment tanks had an average temperature of 21.1 °C but reached an average of 26.1 °C on 18 June, towards the end of the experiment. The temperatures that day exceeded the 26 °C heat tolerance observed by Manis and Claussen (1986) for LISY in New York, further implicating thermal stress as the cause for reduced survival. Further, LISY survival was also reduced by an interaction between temperature and salt such that the combined effect of multiple stressors may be reducing LISY fitness, which has been observed in other studies examining the interactive

effects of climate change (Rohr and Palmer, 2013; Rumschlag et al., 2014). LISY mass at metamorphosis was also related to both elevated salt and elevated temperature treatments independently, with both treatments increasing mass at metamorphosis. As previously mentioned, this response is likely due to increased availability of foraging resources (Van Meter et al. 2011; Petranka and Francis, 2013; Baldwin et al., 2014; Dananay et al. 2015). The elevated chlorophyll a, orthophosphate concentrations, and dissolved oxygen concentrations we observed in the elevated salt treatments further support an indirect effect of road salt increasing periphyton growth within our salt treated tanks. However, despite warm temperatures having the potential to increase biomass and productivity (Baldwin et al. 2014), neither chlorophyll a, orthophosphate concentrations, or dissolved oxygen increased under elevated LISY temperature treatments.

A 3°C mean increase in temperature throughout larval development caused early emergence for both species; however, an interaction between salt and temperature increased development time for both species. The early emergence of our LISY and PSCR tadpoles is consistent with previous literature examining the plasticity of metamorphosis to environmental factors such as temperature and hydroperiod (Wilbur and Collins, 1973; Newman, 1992; Laurila and Kujasalo, 1999). However, a recent study found that warmer winters in the region are likely to cause early breeding, which results in a cooler, not warmer, environment for larval development and thus a longer developmental period with increased growth (Benard, 2015). The study goes on to suggest that even if warming in the region is concentrated during the winter months instead of during larval development, adverse affects in the form of reduced fecundity from increased energy consumption during the winter could still impact the persistence of amphibian populations in the long-term (Benard, 2015). In contrast, it has been found that even when tadpole development is prolonged following early spawning, metamorphs from these early

spawning events still emerge earlier than tadpoles from late reproductive events (Reading and Clarke, 1999). We suggest further experimental comparisons of these differing climatic scenarios in the future to better elucidate this relationship. In addition to early emergence under the elevated treatments, we also found an interaction between road salts and elevated temperature that increased development time for both LISY and PSCR. We suspect that altered osmoregulatory and physiological processes under the road salt treatments slowed development such that individuals exposed to elevated temperatures developed at a slower rate that was more similar to individuals from ambient air treatments.

Ultimately, we encourage a reduction in and more strategic deposition of road salts to minimize impacts to wetlands and wetland fauna. In particular, the use of road salts in areas adjacent to wetlands and other freshwater ecosystems should be minimized whenever possible. We also suggest monitoring amphibian populations for phenological shifts such as early emergence of metamorphs because they can be indicators of climate-related impacts to these populations. Lastly, we stress the consideration of multiple independent stressors and synergistic interactions between them when contemplating how best to reduce threats to amphibian populations in the future.

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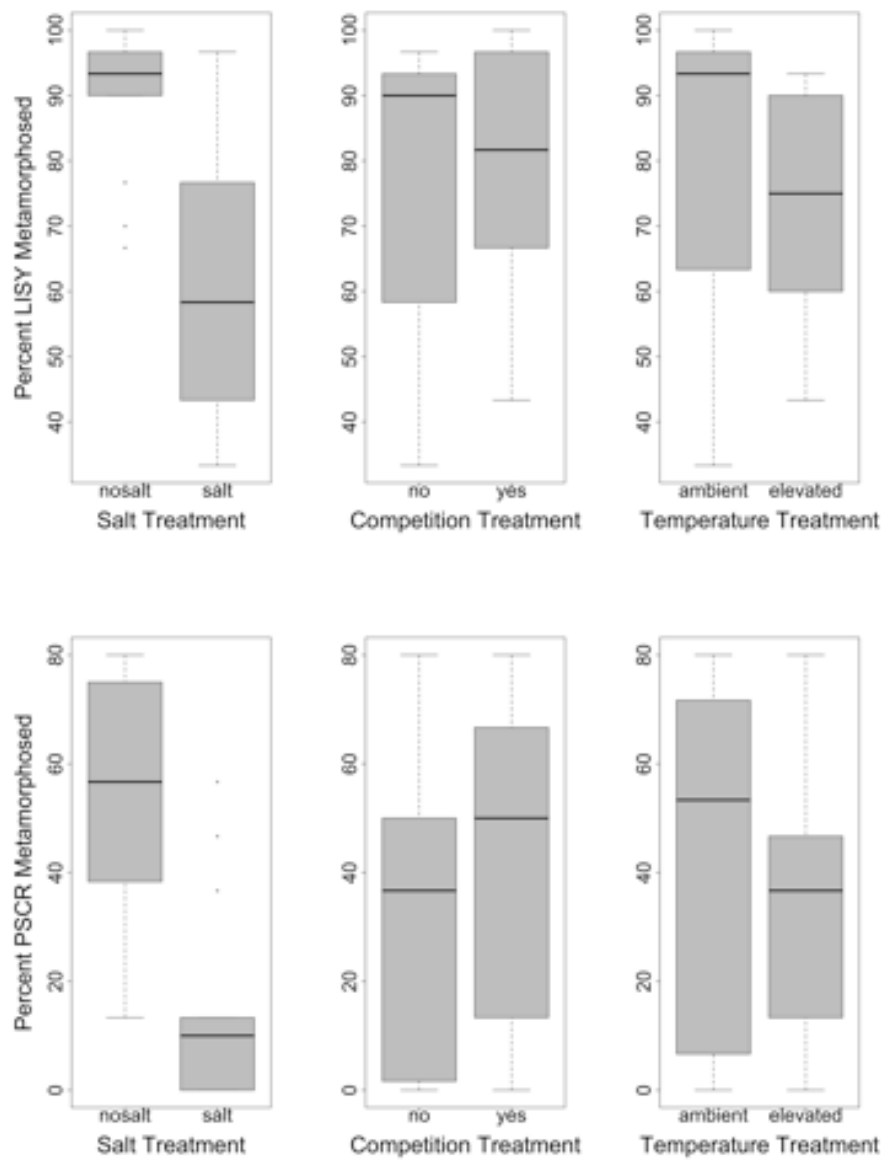


Figure 1. The percent of LISY and PSCR tadpoles surviving to metamorphosis under the various treatments.

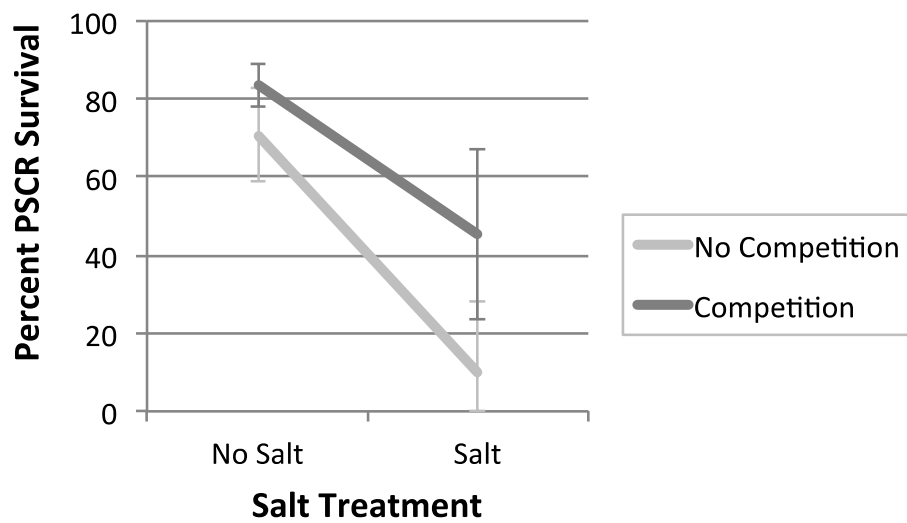


Figure 2. Interaction plot between salt and competition treatments for percent PSCR survival.

Error bars are represented as one SD from the mean.



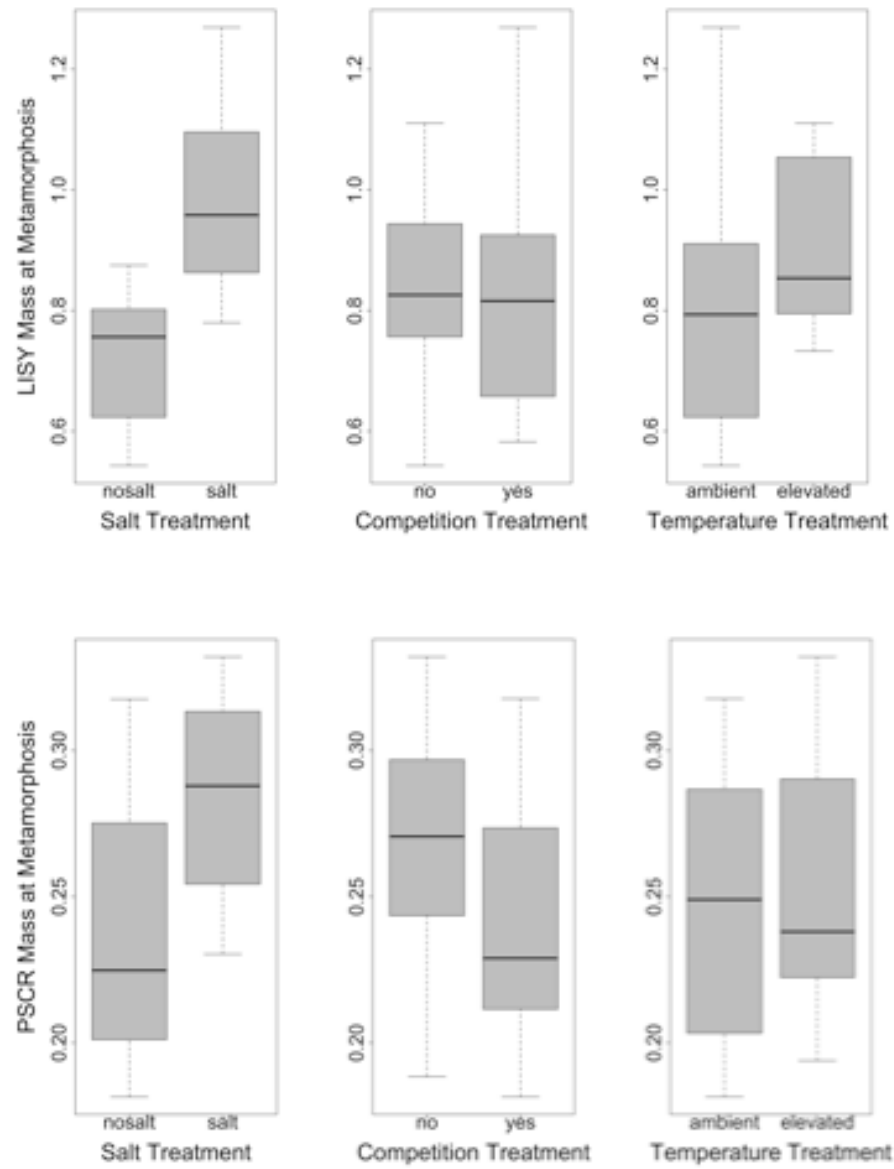


Figure 3. The average mass at metamorphosis (g) for LISY and PSCR under the various treatments.

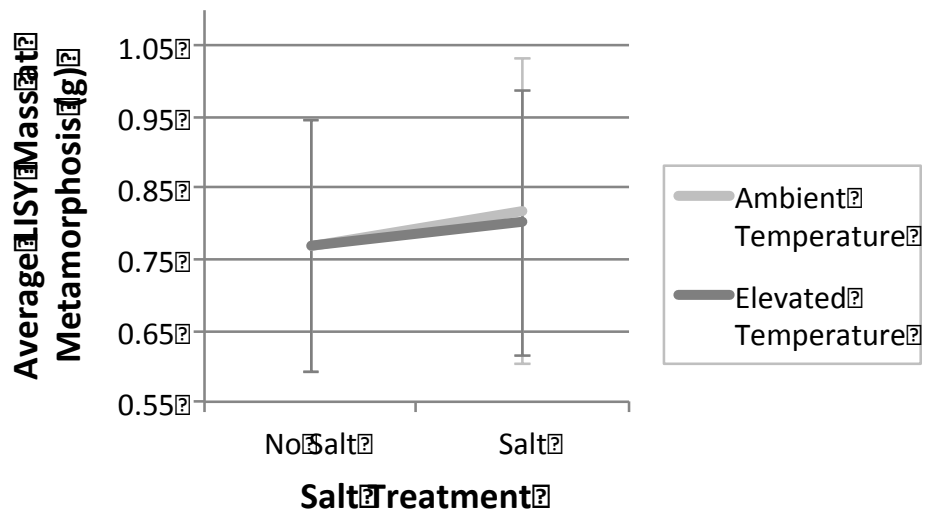


Figure 4. Interaction plot between salt and temperature treatments for average LISY mass at metamorphosis. Error bars are represented as one SD from the mean.

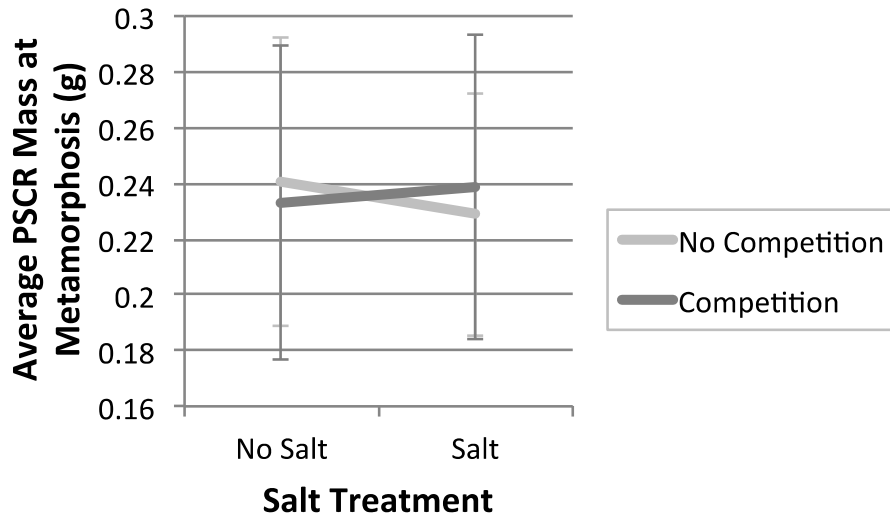


Figure 5. Interaction plot between salt and competition treatments for average PSCR mass at metamorphosis. Error bars are represented as one SD from the mean.

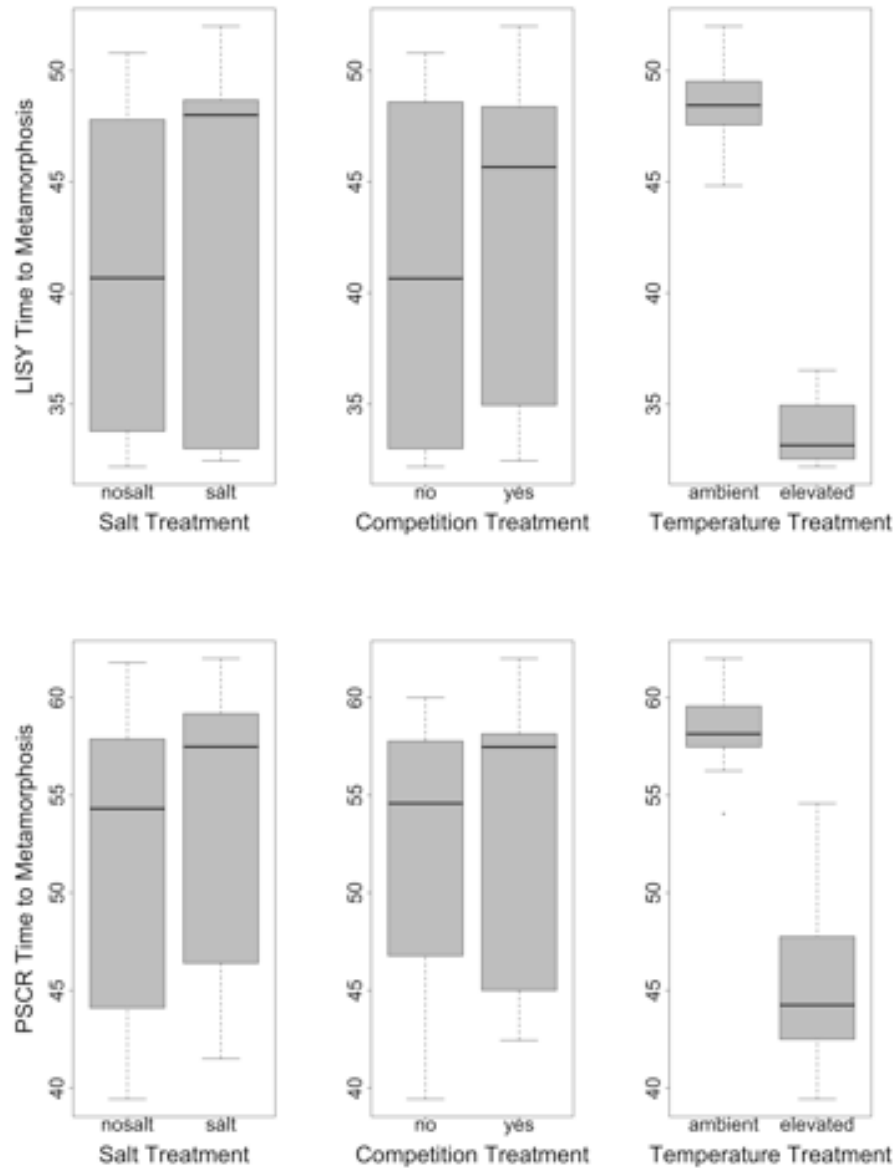


Figure 6. The average days to metamorphosis for LISY and PSCR tadpoles under various treatments.

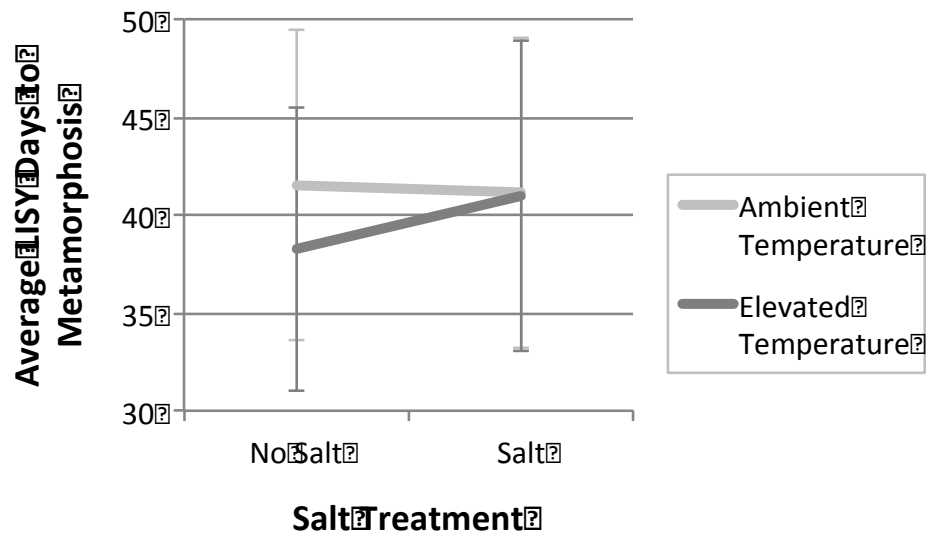


Figure 7. Interaction plot between salt and temperature treatments for wood frog average days to metamorphosis. Error bars are represented as one SD from the mean.

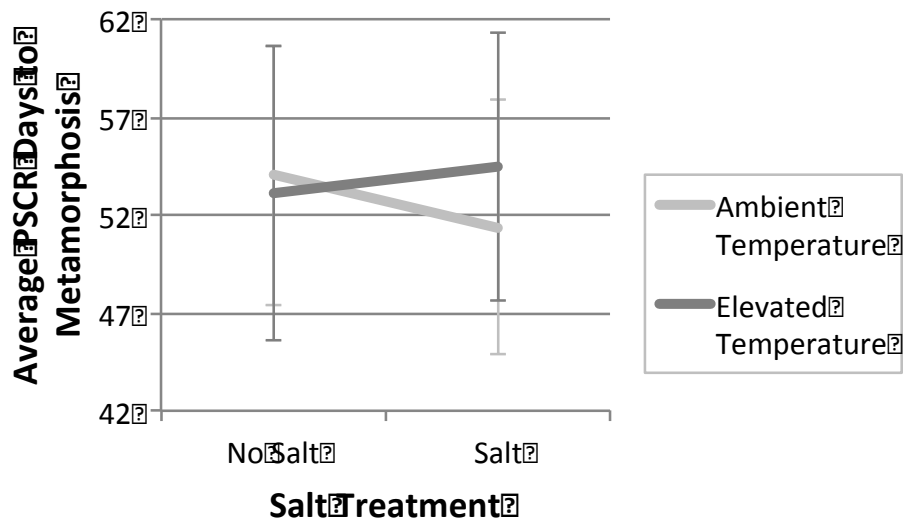


Figure 8. Interaction plot between salt and temperature treatments for spring peeper average days to metamorphosis. Error bars are represented as one SD from the mean.

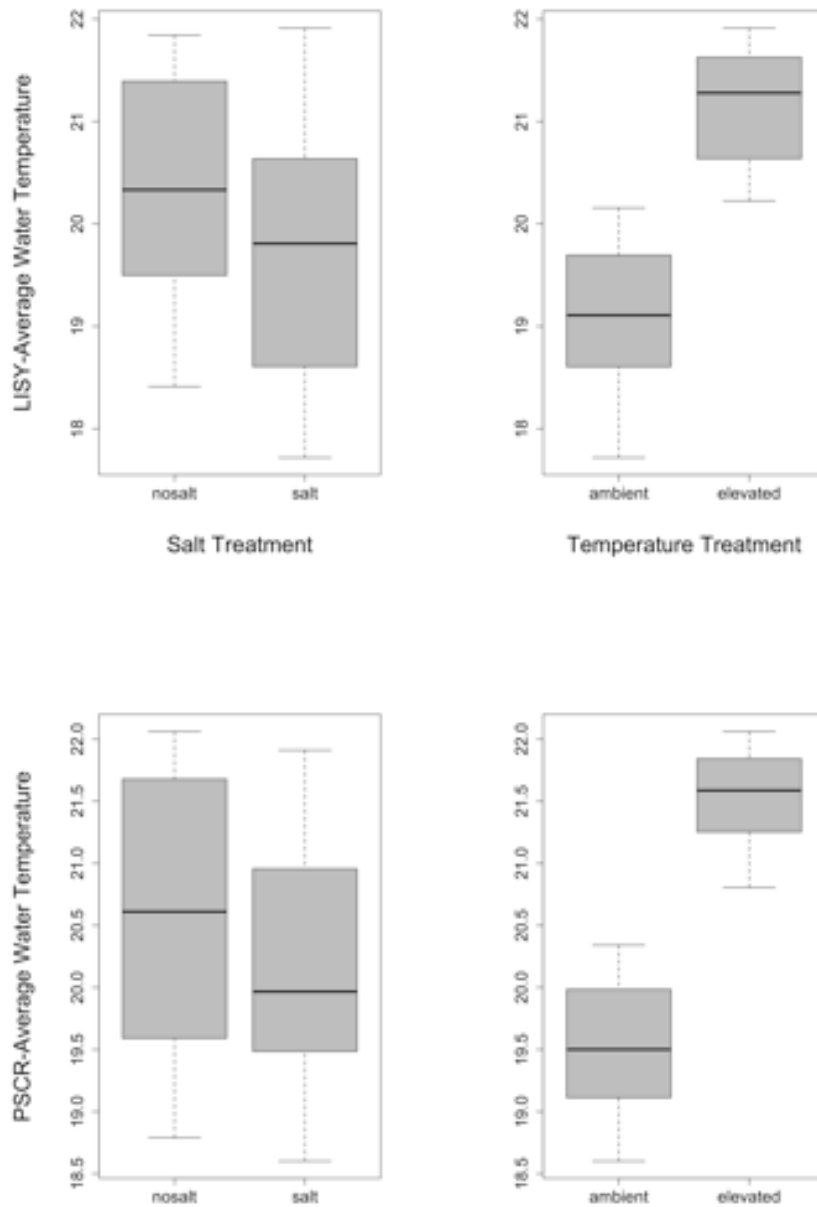


Figure 9. Average LISY and PSCR tank water temperatures (°C) for salt and temperature treatments.

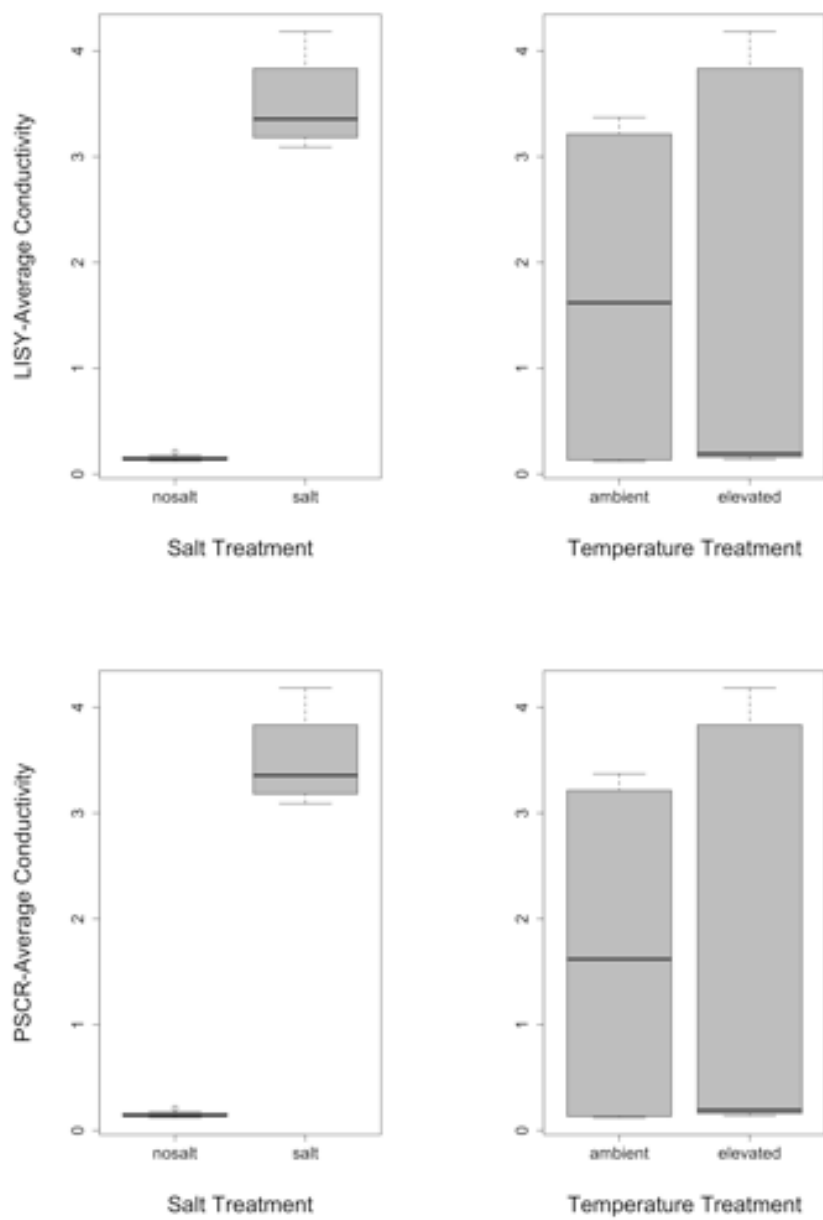


Figure 10. Average LISY and PSCR tank specific conductivity ( $\mu\text{S}/\text{cm}$ ) for salt and temperature treatments.



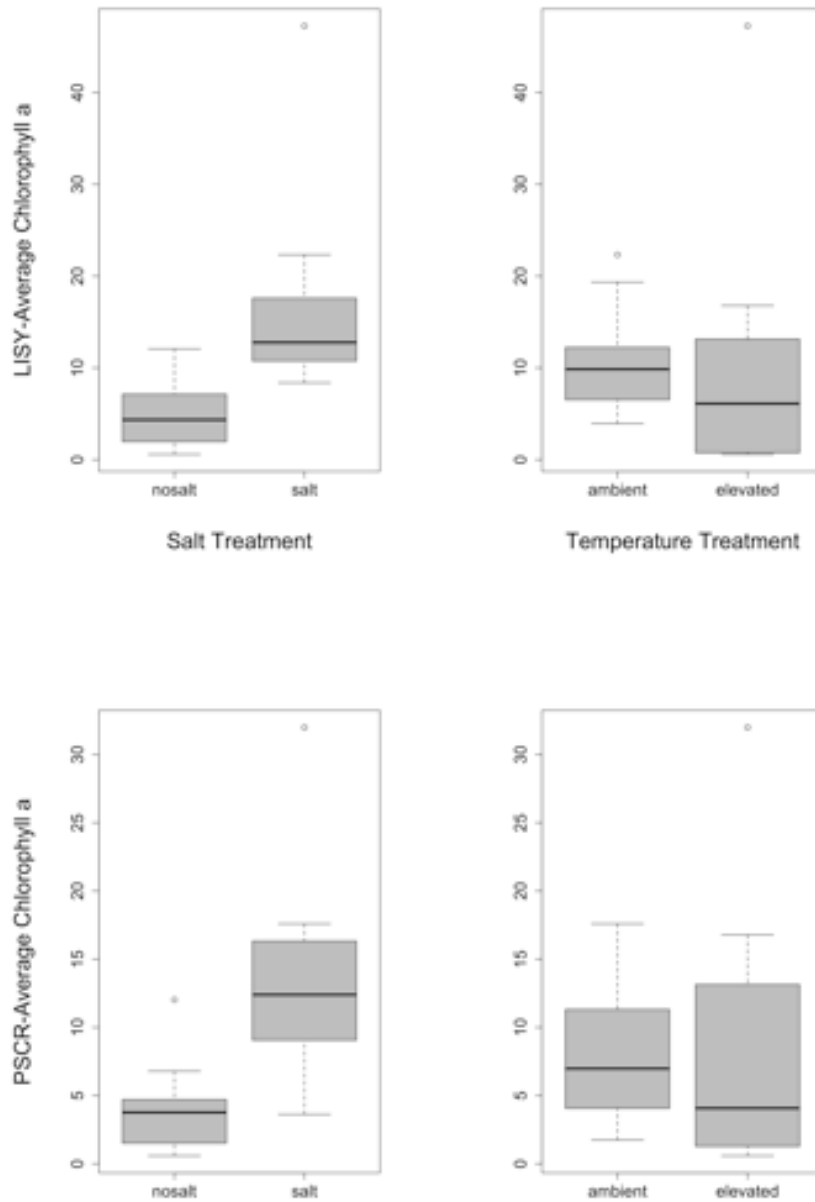


Figure 11. Average LISY and PSCR tank chlorophyll a concentration (mg/L) for salt and temperature treatments.

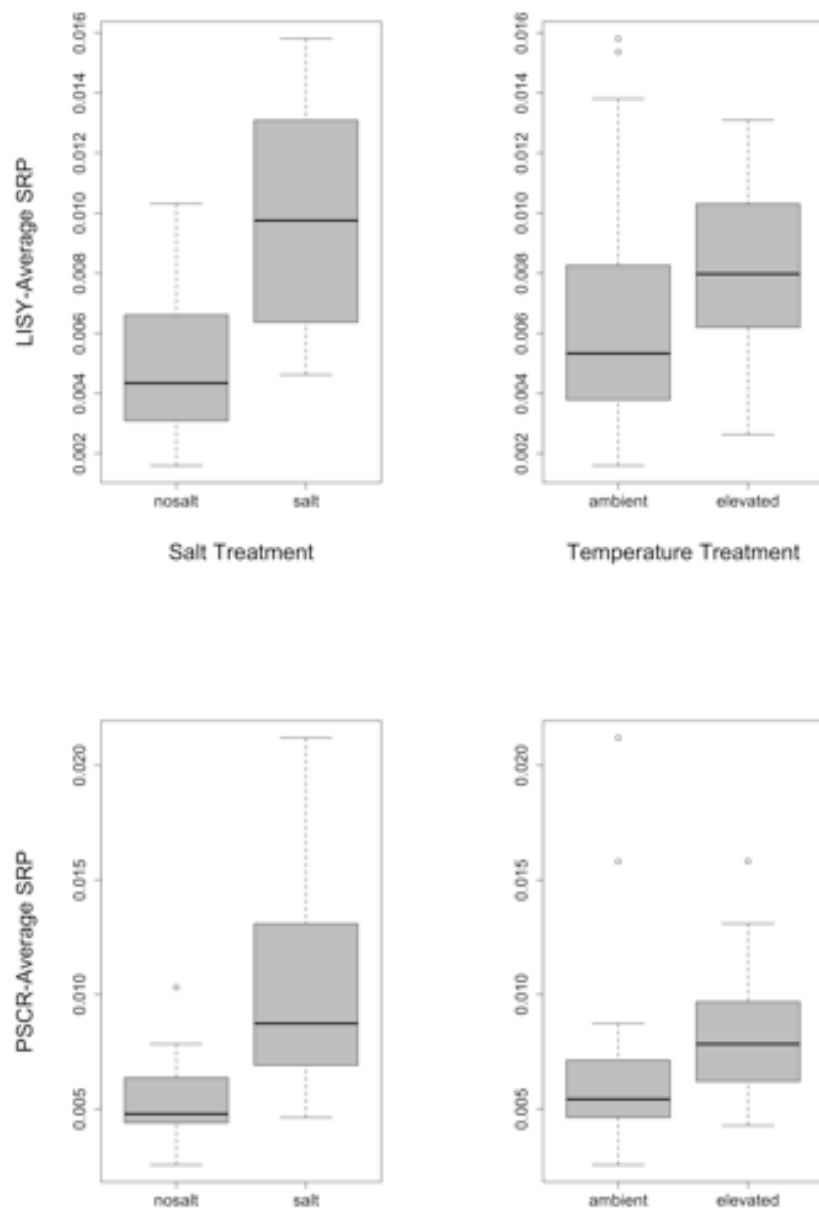


Figure 12. Average LISY and PSCR tank soluble reactive phosphorus concentrations (mg/L) for salt and temperature treatments.

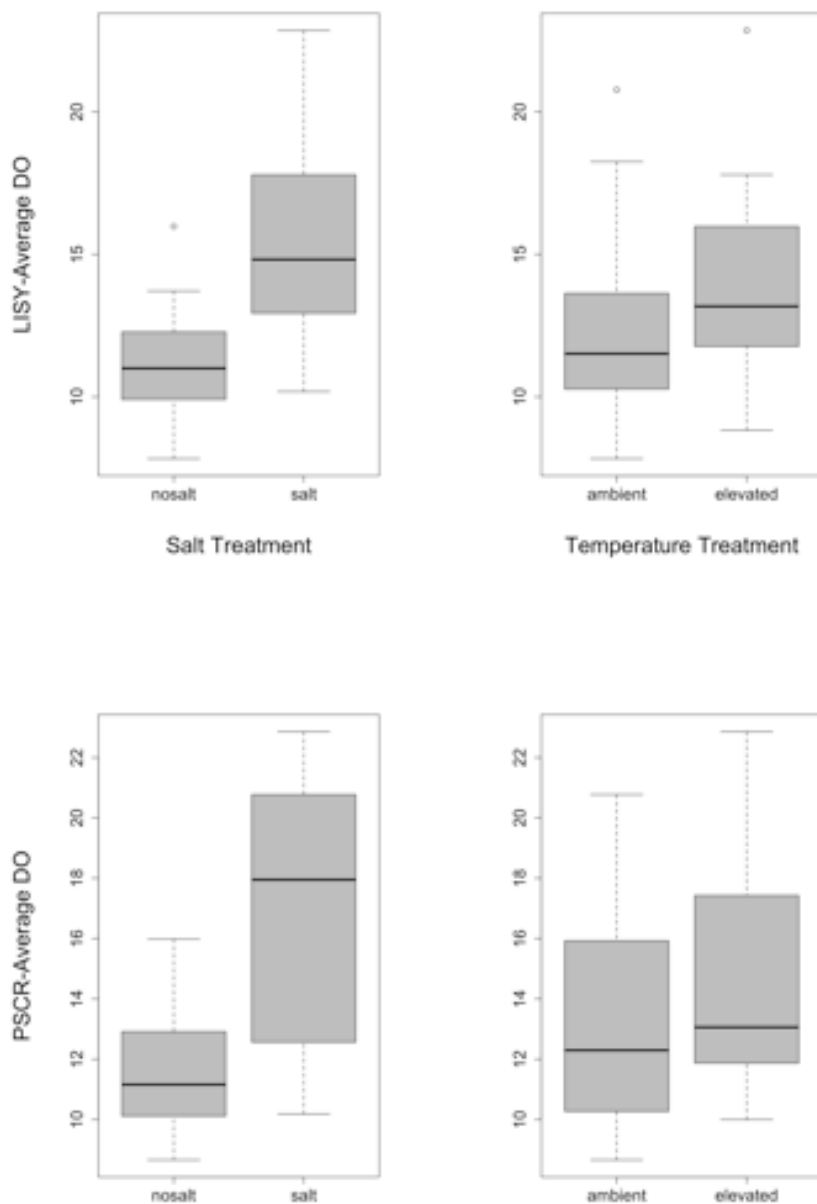


Figure 13. Average LISY and PSCR tank dissolved oxygen concentrations (mg/L) for salt and temperature treatments.