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Population origin and heritable effects mediate road salt toxicity and thermal stress in an amphibian

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HIGHLIGHTS

- Elevated temperature exacerbates road salt toxicity.
- Salt pollution can erode genetic diversity in wild populations and constrain adaptation.
- Populations with a lineage of road salt exposure are more sensitive to its negative effects.
- Multi-stressor studies are critical to understand pollution and climate change.

GRAPHICAL ABSTRACT

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ABSTRACT

Human impacts on wild populations are numerous and extensive, degrading habitats and causing population declines across taxa. Though these impacts are often studied individually, wild populations typically face suites of stressors acting concomitantly, compromising the fitness of individuals and populations in ways poorly understood and not easily predicted by the effects of any single stressor. Developing understanding of the effects of multiple stressors and their potential interactions remains a critical challenge in environmental biology. Here, we focus on assessing the impacts of two prominent stressors associated with anthropogenic activities that affect

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Maladaptation
Pollution

many organisms across the planet – elevated salinity (e.g., from road de-icing salt) and temperature (e.g. from climate change). We examined a suite of physiological traits and components of fitness across populations of wood frogs originating from ponds that differ in their proximity to roads and thus their legacy of exposure to pollution from road salt. When experimentally exposed to road salt, wood frogs showed reduced survival (especially those from ponds adjacent to roads), divergent developmental rates, and reduced longevity. Family-level effects mediated these outcomes, but high salinity generally eroded family-level variance. When combined, exposure to both temperature and salt resulted in very low survival, and this effect was strongest in roadside populations. Taken together, these results suggest that temperature is an important stressor capable of exacerbating impacts from a prominent contaminant confronting many freshwater organisms in salinized habitats. More broadly, it appears likely that toxicity might often be underestimated in the absence of multi-stressor approaches.

1. Introduction

Environmental change threatens biodiversity and poses a multidimensional challenge to conservation. Pollution, climate change, sea level rise, invasive species, and habitat conversion continue to create novel and stressful conditions across the planet. Though often studied piecemeal, effects of these disturbances rarely occur in isolation. Rather, wild populations encounter suites of changes, often concomitantly, with potential to generate complex interactions (e.g., synergisms or antagonisms) occurring in both ecological and evolutionary time. Exposure to stressors can cause lethal and sublethal effects that depress populations. Spatial and temporal heterogeneity in multi-stressor suites can further drive evolutionary population divergence (reviewed in Wadgymar et al., 2022), knowledge of which can be critical for conservation management (Meek et al., 2023). Yet we generally lack understanding of how populations respond to multiple stressors in both evolutionary and ecological contexts (Schäfer and Piggott, 2018). On one hand, multiple stressors might deteriorate niche space and prevent populations from attaining minimal fitness requirements, spurring decline and elevating extinction risk before populations might adapt and recover (Carlson et al., 2014). Or, multiple stressors acting as agents of selection might produce well-adapted populations and/or generalist traits (White and Butlin, 2021; Mérot, 2022; White et al., 2022). Populations can also respond adaptively to stressors through intra or inter-generational plasticity (e.g., Relyea et al., 2023), which can delay or prevent population decline and thus enhance opportunity for selection to drive adaptation (Charlesworth, 2009). Estimating selection gradients for multiple stressors – and understanding whether they constrain or enhance adaptive potential for individual stressors and vice versa (reviewed in Valladares et al., 2007) – is of course complicated by the existence of numerous, potentially interacting selective agents (Crain et al., 2008; Byrne and Przeslawski, 2013; Gunderson et al., 2016; Koch and Guillaume, 2020).

Nowhere, perhaps, is the imperative to understand interacting effects of multiple stressors more pressing than in aquatic habitats receiving non-point source pollution, where diverse suites of contaminants can produce many potential interactions. Further, because contaminants often exhibit temperature-dependent toxicity (Vergauwen et al., 2013; Pereira et al., 2017), climate change is likely to mediate these interactions. Indeed, global lake surface temperatures have risen 0.34 °C per decade (Woolway et al., 2020) and many lakes are projected to shift to lower-latitude thermal regimes (Maberly et al., 2020). Smaller surface waters such as vernal pools or temporary ponds are particularly vulnerable to runoff pollution because their low water volumes can lead to high contaminant concentrations. These habitats are of course also susceptible to warming from climate impacts. Here again, low water volume increases vulnerability as these habitats have little thermal inertia to resist warming. For instance, a recent study of small, temporary ponds in the northeastern U.S. reported an increase in water temperature of 0.22°C over the past two decades (Arietta and Skelly, 2021). Despite the prevalence of multiple stressors on wild systems, studying their impacts is challenging, requiring cumbersome experiments to test numerous treatments and their interactions (Ormerod et al., 2010; Orr et al., 2020). Understandably, many experimental approaches have thus relied heavily on traditional exposure testing, where stressors such as contaminants are tested individually and acutely, often in laboratory settings and not replicated across populations. Unfortunately, these aspects prevent robust inference into multiple stressor effects on ecological and evolutionary outcomes in nature.

Among sources of pollution, roads and parking lots deserve close consideration. The global road network is vast, dispatching 40 million paved and unpaved lane kilometers across the planet (Central Intelligence Agency, 2022). Parking lots cover an additional 5.5% of U.S. metropolitan areas (Falcone and Nott, 2019). Collective impacts of these surfaces extend 20 times beyond their physical footprint (Forman and Deblinger, 2000). Pollution from roads and parking lots shuttles an array of contaminants into terrestrial and aquatic habitats and ground waters. Hydrocarbons (from fuels, lubricants, and pavement) and heavy metals (from vehicle wear and tear) are notable for their sublethal, toxic, carcinogenic, and even mutagenic effects (Huberman et al., 1976; Kapitulnik et al., 1977). In regions with cold winters, deicing salt is one of the most common road pollutants. In the U.S., about 24.5 million tons of salt are applied annually to deice paved surfaces, triple the amount applied four decades ago (Bolen, 2019). Salt pollution from deicing has caused the salinization of many freshwater habitats and drinking water sources across North America (Dugan et al., 2017; Kelly et al., 2019; Van Meter and Ceisel, 2021; Solomon et al., 2023). Much of the road salt research has focused on lake and riverine systems. Smaller waters like ponds and wetlands have received less attention yet can reach especially high salinity, at times containing concentrations typical of saltwater estuaries (e.g., Brady, 2012).

Roads are not the only source of salt pollution. Many freshwater habitats are becoming salinized through intrusion from sea-level rise and runoff from agricultural practices and mining effluent (Kaushal et al., 2018). Likewise, road impacts are not limited to pollutants, but also include fragmentation, road kill (Ibrahim et al., 2018), and the spread of invasive species and pathogens (Urban, 2006; Hall et al., 2020; Numminen and Laine, 2020). Ultimately, road effects can lead to population declines, local extinction, and changes in community structure and ecosystem function (Forman and Alexander, 1998; Coffin, 2007; Hintz and Relyea, 2019). Along the way, and seldom considered, these numerous agents of change caused by roads also impose novel agents of natural selection (Brady and Richardson, 2017).

Many freshwater aquatic organisms have shown sensitivity to salinization (Karraker et al., 2008; Brady et al., 2017; Hintz and Relyea, 2017, 2019; Sinclair and Arnott, 2018; Arnott et al., 2020). Here, we conducted a suite of experiments and assays to further our understanding of the adverse effects of road salt pollution on an aquatic organism vulnerable to salinity stress and rising water temperatures, the wood frog (*Rana sylvatica*). We present a series of investigations aimed at unveiling some of the complexity and context-dependency of such adverse effects, including variation across developmental stage, the effects of interacting stressors, and transgenerational effects mediated by ecological and evolutionary forces. For Nearctic amphibians like wood frogs, climate change followed by habitat degradation (e.g., from pollution) are thought to pose the most significant risks to populations

(Luedtke et al., 2023). In the context of climate change and pollution, rising water temperatures (Barbarossa et al., 2021) and freshwater salinization (Kaushal et al., 2018, 2021) are threatening the success of amphibian populations that depend critically on aquatic habitats. Little is known about how warmer waters might interact with freshwater salinization to influence amphibians, though recent evidence suggests that larval exposure to elevated temperature and salinity can generate negative carry-over effects on juveniles after metamorphosis (Dahrouge and Rittenhouse, 2022).

Previous work in our study system has shown that local populations of wood frogs can differ substantially in tolerance to road salt. Surprisingly, differences between populations seemingly contradict local adaptation predicted by natural selection, suggesting that polluted populations might be relatively maladapted to pollution. Specifically, our initial expectations were that wood frogs breeding and developing in salt-polluted ponds should be locally adapted, showing higher tolerance to polluted ponds (as has been shown for a cohabiting species of amphibian, *Ambystoma maculatum* (Brady, 2012)) and to experimental salt exposure. Through a series of highly replicated field transplant and laboratory exposure experiments, we found that neither of these expectations held. In fact, compared to populations from unpolluted ponds, road-adjacent populations with a lineage of exposure to pollution survived at lower rates, both in road-adjacent ponds (Brady, 2013, 2017) and in common garden road salt exposure experiments (Brady et al., 2017; Forgione and Brady, 2022). Despite this overall survival disadvantage, wood frog populations harbor genetic variation, including genotypes with high embryonic survival in high-salinity environments (Brady and Goedert, 2017). Thus, populations appear to have capacity for adaptation, and/or tradeoffs across life history stages might mediate responses to road salt pollution. Indeed, in addition to a suite of maladaptive processes (see Brady et al., 2019a; Brady et al., 2019b) that might explain embryonic survival disadvantages in roadside populations, reduced embryonic survival might tradeoff with traits that increase fitness at later life history stages. Most recently, we have learned that across aquatic stages, maladaptive survival is limited to embryonic developmental stages, beyond which larvae from polluted ponds no longer experience a survival disadvantage, either in common garden or reciprocal transplant experiments (Forgione and Brady, 2022). Thus, any potential tradeoffs governing this pattern might be found in adult traits. Indeed, gravid females from roadside populations jump further (Brady et al., 2019c) and lay more eggs (Brady, 2013; Brady et al., 2019c) than their woodland counterparts.

Motivated by the finding that maladaptive survival is limited to embryos, we conducted an exposure experiment to characterize embryonic survival curves and developmental patterns asking whether developmental stages are differentially sensitive to salinity stress resulting from road salt. Contrary to multiple previous experiments, the results of this experiment (reported herein) showed no effect of salt on survival, and all malformations induced by salt resolved prior to larval feeding stage. Unseasonably low temperatures during that experiment prompted us to consider whether thermal stress might interact with osmotic stress from salt. We therefore asked whether critical thermal maximum might differ for tadpoles based on their history of salinity stress during development (i.e. roadside versus woodland ponds, respectively). We also conducted an experiment to investigate interacting effects of temperature and salt on life history and physiological traits across a wide range of ecologically relevant salinity and temperature values. Finally, motivated by past maladaptive survival patterns, and to aid our interpretation of differences in responses to salinity stress across population types, we investigated how underlying mechanisms of population origin and salinity exposure affect salinity sensitivity. To test these mechanisms, we first conducted a common garden exposure experiment to investigate differences in family-level variance for survival and days to mortality between high and low salinity treatments for both population types. In this experiment we asked if population type explains differences in mean salinity sensitivity and whether reduced genetic variance and/or beneficial transgenerational effects under high salinity could explain constrained adaptive responses to salt pollution. Next, we compared estimates of osmotic stress across population type and salinity exposure treatments. Finally, to place our results in the context of conditions found in wild populations and to better understand adverse effects of roads on amphibian populations, we examined the relation between salinity, temperature, and dissolved oxygen for a suite of ponds distributed across a pollution gradient. Taken together, the suite of results presented here help uncover the complexity in physiological and (mal)adaptive responses to salinity stress while paving new directions for future research.

2. Methods

2.1. Natural history and study sites

Wood frogs are medium-sized Anurans with a range spanning much of Canada and eastern U.S. (Martof and Humphries, 1959; Green et al., 2014). Although highly terrestrial after metamorphosis, adults are commonly found near ponds used for breeding in spring. Across our study area, breeding typically occurs between early March and mid-April, lasting about 1–2 weeks in any given pond. Adults migrate from uplands to mate in ephemeral ponds. During breeding, males amplex females and fertilize eggs externally upon oviposition. Each female lays one egg mass with about 800–1100 eggs. Embryos develop over 2–3 weeks (depending in part on water temperature) before hatching and continue to develop as larvae until they metamorphose into terrestrial juveniles, typically in mid-summer, before dispersing into upland habitats. Adults can live for 5–6 years (Berven, 2009; Brady et al., 2019c), and apart from annual breeding, tend to spend most of their lives in terrestrial habitat. Each breeding pond is generally considered a population, with low dispersal (13–20%) typically occurring in the juvenile stage, and almost complete philopatry among adults for breeding sites (Berven and Grudzien, 1990).

Among experiments reported here, wood frog embryos and/or larvae were collected from a total of 16 ponds across two different study sites in northeastern U.S., located either in southern Connecticut or central Vermont (Fig. 1). Following Brady (2013), ponds were categorized as either 'roadside' (<15 m from a paved road) or 'woodland' (>150 m from any road). Previous work coupled with conductivity (a close proxy for road salt) values reported here show that, compared to woodland ponds, roadside ponds are heavily polluted with road salt (and typically contain a suite of other runoff contaminants such as plastics, tire particles, and heavy metals). Woodland ponds have little to no pollution from runoff. For instance, conductivity declines exponentially with distance from roads, reaching background values 170 m away from the road (Karraker et al., 2008). Thus, we selected ponds to include heavily polluted ponds adjacent to roads and ponds that appear to receive no road salt inputs (Brady et al., 2019c, 2022).

2.2. Salinity effects across developmental stages

To test if embryonic developmental stages are differentially sensitive to road salt, we investigated the consequences of salinity exposure on developmental rate, malformation, and survival of tadpoles throughout their embryonic period. In Spring 2021, we conducted a common garden salt-exposure experiment in an outdoor venue in New Haven, CT. Outdoor space was used to incorporate as much natural variation in temperature and lighting as feasible. On 8 April 2021 we collected six wood frog egg masses from each of six ponds in central Vermont (N = 3 'roadside' ponds, 3 'woodland' ponds; Fig. 1A) within two days of oviposition, selecting the egg masses that appeared to be laid most recently (i.e., have taken up the least amount of water). Specific conductivity was measured at each pond during embryo collections to verify road salt pollution. Each egg mass is typically sired entirely by one male and thus each egg mass generally represents one full sib family

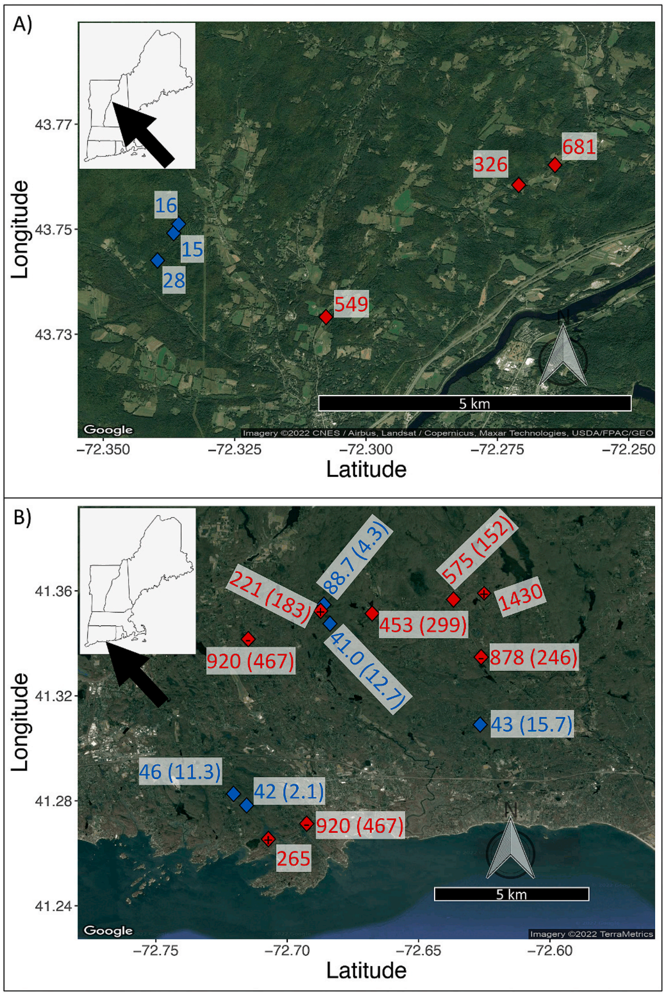

Fig. 1. Location of source populations. (A) Ten ponds (N = 5 roadside, N = 5 woodland) in southern Connecticut and (B) six ponds in central Vermont (bottom panel) were used in the suite experiments. Red symbols indicate roadside ponds while blue symbols indicate woodland ponds. In the Southern Connecticut study site, three of the five roadside ponds (indicated with minus signs) used in the exposure experiment later produced no larvae during dipnet collections for the CT max experiment and three additional ponds (indicated with plus signs) were sourced. Inset shows New England, with each study area approximaltey indicated. Conductivity values (±1 SD where multiple measures were taken) are shown beside each pond.

(Halverson et al., 2006). We transported egg masses to the lab in New Haven, CT, where they were stored with ice packs in coolers overnight until stocking in the outdoor venue the following day. Each egg mass was scored for Gosner developmental stage (Gosner, 1960) immediately before stocking. From each egg mass, we carefully removed six fertilized embryos, assigning three to low salinity treatments and three to high salinity treatments. Treatments were made with aged, conditioned tap water. No additions were made to the low salinity treatment, which had an estimated background concentration of 51.5 mg/L Cl-as reported in the annual water quality report from the Regional Water Authority that supplies drinking water to our lab (Regional Water Authority, 2022). Although this value is higher than what we typically find in woodland ponds (10–30 mg/L Cl-), it is still considerably lower than concentrations found for roadside populations, which can reach conductivity levels that are 14–60 times that of woodland populations (Brady et al., 2022, Brady et al. 2019a,b,c). Moreover, our previous laboratory experiments using the same water source have produced high wood frog survival rates to metamorphosis under identical low salinity conditions (Forgione and Brady, 2022), thus providing a suitable representation of low salinity environments. For high salinity, we added NaCl road salt (obtained from the Connecticut Department of Transportation) to titrate a concentration of 1078 mg/L Cl-, corresponding to a specific conductivity value (measured with a YSI Pro DDS handheld meter) of 4000 μS/cm. This value represents the highest concentration of Cl-we have observed in wood frog breeding ponds in our study region. (Identical titration methods were used to create additional chloride treatments in section 2.4 below.) Titration details and the relation between Cl-concentration and specific conductivity are shown in a workbook file in Supplementary Materials.

Embryos were stocked into a single 17 mL well on a six-well plate where each plate contained one embryo from each of six families collected from a given pond (1 embryo per family X 6 families per pond = 6 embryos per plate). A total of 216 individuals were stocked (6 ponds x 6 families per pond x 2 treatments x 3 embryos per treatment = 216). All wells in each plate were filled completely with the same type of treatment water. Lids were placed on well plates preventing evaporative loss (and thus water was not renewed during the experiment), which were secured with rubber bands. Plates were floated in groups of three within clear 5.5 L plastic containers (30.5 X 20.3 × 10.8 cm) filled with 4 L of like treatment water to act as a water bath, stabilizing water temperature within well plates. These bath containers were then covered with clear, loose-fitting lids and placed on one of three shelves on a resin shelving unit. On each shelf, we placed two low salinity and two high salinity containers from each of two ponds (one roadside and one woodland). Every 1–2 days, we viewed embryos under a dissecting stereomicroscope to record developmental stage, axial malformations, and day of mortality. We screened for malformations following Bantle et al. (1991), however we included observed malformations that were not previously described (e.g., herniated yolk plug) and included a more nuanced assessment of axial malformations (see Supplementary Table 1), which appear to be the most common form on malformation in developing anurans exposed to salinity stress (Karraker, 2007). Malformations were scored as present/absent according to the type of malformation observed. Because mortality in embryos is not immediately obvious (e.g., early-stage embryos do not respond to physical stimulus), day of mortality was determined retroactively by an embryo's failure to advance to the next developmental stage. We ended the experiment after 17 days on 26 April 2021, when all surviving individuals reached hatchling stage (i.e., Gosner stage 25). We monitored water temperature every 30 min throughout the experiment using deployed loggers.

Statistical analyses (here and throughout) were conducted in R (V. 4.2.1) (R Development Core Team, 2022). We first analyzed developmental stage at stocking to test for difference between population types at the onset of the experiment. We used the 'lme4' package (Bates et al., 2015) to compose a linear mixed model with pond of origin as a random effect and population type as the main effect. We then used a mixed model to analyze developmental stage as a function of time. Prior to fitting a model, we inspected a scatterplot with a loess function and observed a pattern suggesting that developmental rate potentially varied as a second or third order polynomial as a function of time. We therefore began by fitting a model containing a third order polynomial for 'day' interacting with salinity as fixed effects, with the plan to remove any non-significant polynomial terms from the models used for inference. In the least parameterized model, we included random effects for pond of origin and individual (because each individual was measured multiple times through the experiment). We used AIC to compare this minimally parameterized model with two additional models that included random effects terms for either family or experimental well plate.

Next, we analyzed the presence of malformations as a function of developmental stage and population type. Because no malformations were detected in low salinity treatments, we did not include salinity as a term in the analysis, but instead limited our analyses to the high salinity treatment. However, we evaluated the confidence intervals for the intercept to evaluate whether the prevalence of malformations in high salinity differed from zero. We composed a generalized linear mixed model with a binomial response (1 = presence of one of more malformations, 0 = no malformations). Initial plotting of the presence of malformations in relation to developmental stage suggested a quadratic pattern. For fixed effects, we therefore modeled the presence of malformation as a function of a second order polynomial for developmental stage and its potential interaction with population type. To select the corresponding random effect structure, we composed a suite of candidate models varying in random effects, containing all combinations of additive terms for individual, family, and population (with population retained in the most minimal model). We selected for inference the model best supported by AIC procedure, where lower AIC values correspond to relatively better-fit models. Specifically, AIC was calculated for all models. We then selected the model with the lowest AIC that was at least 2 AIC units (i.e., delta AIC >2) more negative than the next best model (Burnham and Anderson, 2004). If interactions between fixed effects were not supported by log-likelihood assessment, models were refit without the interaction term before repeating selection procedures for random effects and subsequent inference of the selected model. Separately, we used Cox regression to analyze survival over time and in response to population type and salinity, using the 'coxph' function in the R library 'survival' (Therneau, 2015).

2.3. CT max in wild tadpoles from salinized vs. unsalinized ponds

On 2 May 2022, we used hand dipnets to collect a target of 10 tadpoles from each of 10 ponds in CT (five roadside, five woodland) with the goal of assessing critical thermal maximum (CT max). We sampled 10 ponds, five roadside and five woodland ponds that were also used for a separate exposure experiment described below in section 2.4. Dipnet efforts produced no tadpoles in three of those ponds and we therefore sampled three additional ponds (Fig. 1B). Tadpoles were collected into 700 mL plastic containers filled with natal pond water and brought to the lab. Tadpoles were housed in these containers for two days under ambient laboratory conditions of about 22^{o} C with a 14:10 h light cycle. To measure CT max (sensu Chuang et al., 2022; Cicchino et al., 2023), tadpoles were individually placed into a 250-mL beaker containing aged and conditioned tap water housed within a room temperature water bath. After an initial 10-min acclimation period, we increased the water bath temperature by 0.5 °C per minute. Tadpoles were regularly and lightly touched on the tail using a disposable plastic pipette. CT max was recorded as the temperature when tadpoles ceased responding to stimulus. This process was repeated across six rounds over three days, with each tadpole randomly assigned to one round and ensuring that each population was represented in each round with an approximately equal number of roadside and woodland tadpoles. In total, CT max was measured for 84 tadpoles.

We used the 'lme4' package (Bates et al., 2015) to compose a linear mixed model to test whether CT max varied across the interaction of population type and developmental stage. Two random effect structures were evaluated before inferring potential interacting effects of population type and developmental stage: 1) population, or 2) population and round. AIC selection was applied to choose the model for inference.

2.4. Interacting effects of salinity and temperature on survival

In spring 2022, we used an exposure experiment in the lab to test interacting effects of salinity and temperature on embryonic survival. Complete details and design schematic are reported in Supplementary Materials. Briefly, we selected 36 combinations of temperature and salinity representing a wide range of ecologically relevant conditions. Embryos from 10 different families (i.e., egg masses) from each of 10 different populations (5 roadside, 5 woodland) were collected within two days of oviposition from ponds located in southern Connecticut (Fig. 1B). We selected the freshest looking egg masses to capture the earliest developmental stages and standardize starting stage. Each egg mass was scored for Gosner developmental stage at the start of the experiment. From each pond, 25 embryos were assigned to either a woodland container or a roadside container, corresponding to their population type of origin, for a total of 125 tadpoles (i.e., 25 embryos x 5 ponds per type) per container. Thus, 36 containers housed 125 woodland embryos each while 36 containers housed 125 roadside embryos each. Each of these two sets was exposed to one of the 36 combinations of temperature and salinity, for a total of 72 treatments with 9000 embryos. All embryos were collected on 18 March 2022 and stocked into the experiment on 19 March 2022. Survival was assessed after 10 days of exposure.

We first tested for differences in starting Gosner developmental stage as a function of pond of origin using a linear mixed model with pond as a random effect. We analyzed survival as a function of the three-way interaction between salinity, temperature, and population type using a standard generalized linear model (R function 'glm') with a logit link where survival was a binomial response (number stocked, number survived). Interactions were evaluated with likelihood ratio tests comparing the fully parameterized model containing a three-way interaction with each reduced model containing all iterations of partially interacting or purely additive effects.

2.5. Population and family-level components of salinity tolerance

In spring 2022, we also conducted a chronic salinity exposure experiment in the lab—raising embryos to near-metamorphosis (i.e. Gosner stage 42)—to estimate mean differences in survival and days to mortality between treatments and population types, as well as family-level variance (V_F) and repeatability (i.e., the proportion of total variance attributed to family-level variance: $R = V_F/V_T$) of two traits: survival and days to mortality. V_F is composed of genetic variance (additive and non-additive) and shared environmental effects within families (e.g., transgenerational parental effects). From each of 10 egg masses (described in section 2.4), we carefully separated two additional clusters of five embryos and stocked each into 600 mL of aged, conditioned tap water under low (ca. 51.5 mg/L Cl-, no salt added) or high (ca. 1078 mg/L Cl-) salinity. Road salt obtained from the Connecticut Department of Transportation was used to create the high salinity treatment. After tadpoles reached feeding stage, water was statically renewed every five days, at which time tadpoles were fed 5-day rations of food (3:1 rabbit chow and fish food), which was calculated at a rate of 10% body mass per tadpole per day. Individuals were assessed for mortality every 1–2 days throughout development until the most-developed tadpoles reached Gosner stage 42. At this point, we terminated the experiment (after 48 days), by which time all embryos had either died or reached mid to late larval developmental stages (Gosner stages 27–42). We chose to terminate the experiment at this single time point to avoid the confounding effects of duration exposure on survival and days to mortality. A 14:10 h light cycle was maintained during the experiment and water temperature (monitored every 30 min with deployed HOBO Pendant MX Temperature loggers) averaged 20.3 °C (±1.10 SD).

Using separate models, we analyzed overall survival and days to mortality across the interaction of population type and salinity. Survival was analyzed as a binomial response using a generalized linear mixed effects model with a logit link. Days to mortality was analyzed using a linear mixed effects model. For each response variable, we used AIC to select between three candidate models differing in random effects structures. The first model contained terms for population and family nested within population, the second model contained terms for population and family, the third model contained one term for population. After model selection, a likelihood ratio test was used to assess main effects and their interaction in the model for survival. The Satterthwaite method implemented in the R package 'lmerTest' (Kuznetsova et al., 2017) was used in the model for days to mortality. Interactions were removed if not supported.

We fit separate threshold (i.e., probit) models for survival and days to mortality to estimate V_F and R for each combination of population type (roadside vs. woodland) and treatment (high vs. low salinity), using the MCMCglmm package v. 2.35 (Hadfield, 2010). In the survival model, family was fit as the random effect, whereas the model with days to mortality included the individual identity nested within family. We used parameter extended priors for V_F and fixed the residual variance to 1 (therefore, $V_T = V_F + 1$). Each model was fitted with at least two different starting values to check for convergence, and results were compared with those of models fitted using an inverse-Wishart prior for the random effects. Models ran for >1,000,000 iterations, returning at least 1000 in effective sample sizes. We visually inspected the trace plots of the posterior distributions to check for quality of mixing, and used a Heidelberger and Welch's convergence diagnostic in package 'coda' v 0.19–4 (Plummer et al., 2006). We compared V_F and R for each population type between salinity treatments, and for population type within each treatment, by taking the difference across all iterations of the posterior distributions. Therefore, we obtained a distribution of differences from which we were able to obtain the mean, modes and 95% high posterior density [credible] intervals. All results are reported in the latent scale. Due to large credible intervals, results for survival are presented in Supplementary Materials.

2.6. Osmolality responses across population type and salinity exposure treatments

We assayed larvae from the experiment described above (section 2.5) to examine osmolality responses to salinity stress. Amphibians osmoregulate largely through ion exchange. In freshwater ponds, the environment is hypoosmotic to amphibians, which need to pump ions inward to counter the tendency for ions to be lost through diffusion. However, in the high salinity environment tested here, we expected internal osmolality to increase, potentially because of a high cost and/or physiological limit of maintaining a lower and presumably more optimal internal osmolality. We further asked whether populations differ in their degree of ion uptake, indicating why roadside populations are generally more sensitive to road salt (Brady et al., 2022). We measured body fluid osmolality in a subset of late-stage tadpoles (ranging from Gosner stages 35–40). We haphazardly selected 2–3 tadpoles per population per salt treatment to measure whole body fluid osmolality. We flash froze tadpoles (to avoid potentially confounding effects of chemical-based euthanasia agents) by placing each tadpole in a plastic vial submerged in liquid N for 10 s. Tadpoles were immediately transferred into a 2 mL centrifuge tube, macerated with a handheld homogenizer, and centrifuged for 1 min at maximum speed to separate tissue from body fluid. A 10 μL sample of the liquid supernatant was analyzed in an osmometer (Wescor Vapro® model 5520). In total, 51 tadpoles were assayed for body osmolality (N = 29 low salinity [14 roadside, 15 woodland], N = 22 high salinity [9 roadside, 14 woodland]).

We used the 'lme4' package (Bates et al., 2015) to compose a suite of linear mixed effects models testing for the potentially interacting effects of developmental stage, population type, and salinity. Interaction terms were evaluated using likelihood ratio tests. In all models, population identity was included as the random effect.

2.7. Environmental variation in natural ponds

To evaluate potential covariates associated with road proximity, we measured water quality variables from a suite of southern CT ponds in spring 2020, 2022, and 2023. Specific conductivity (standardized to 25 °C) and dissolved oxygen was measured in 21 ponds (N = 16 roadside, 6 woodland) with YSI Pro DDS or Oakton PCTS Testr™ handheld meters. Temperature loggers (model: HOBO Pendant MX Temperature) were used to measure temperature every 30 min from 24 to31 May 2022 in four roadside and four woodland ponds. All handheld and deployed measurements were taken at the deepest location in the pond (i.e., z-max) at a depth of 10 cm. Temperature loggers were secured by nylon fishing line to an 8 cm^2 square piece of pink, closed cell foam, which contained a cable tie that pierced through the foam to form a girdle through its central region, acting as a secure attachment point. We premeasured the nylon line to achieve a precise logger depth of 10 cm. We tied three or four overhand knots to ensure a secure attachment. The temperature logger, which is embedded in a plastic case, needed to be weighed down to prevent it from floating. For weight, we attached a stainless-steel nut to each temperature logger with another cable tie. Finally, in each pond, the floating portion of a logger was attached loosely with a large loop of cable tie to a wooden stake at z-max, allowing the temperature logger to self-adjust with rising and falling water levels.

We used separate linear mixed models to analyze specific conductivity, dissolved oxygen, and temperature as a function of pond type, with pond identity included as the random effect. We used an additional set of separate linear mixed models to analyze dissolved oxygen and water temperature as a function of conductivity (with pond identity as a random effect) to evaluate whether either of these variables correlated with the intensity of salt pollution. Conductivity was log-transformed in all analyses to improve normality based on preliminary inspection of QQ plots. Degrees of freedom for p-value estimates were calculated with Satterthwaite's approach (R package 'lmerTest'; Kuznetsova et al., 2017).

3. Results

3.1. Salinity effects across developmental stage

In the context of the outdoor-based embryonic salinity exposure experiment, developmental stage of embryos collected from the wild did not differ between population types at the onset of the experiment ($F_{1, 4} = 0.220$, $P = 0.664$). Across all individuals, initial Gosner developmental stage was estimated to be 9.97 (95% CI = 9.63 to 10.32). For the analysis of developmental stage over time, the most parameterized model (containing random effects for pond of origin, individual, family, and experimental well plate) was selected for inference (delta AIC >2.90). Developmental stage varied as a third-order polynomial function of time ($F_{1, 2371} = 159.8$, $P < 0.001$), which did not interact with salinity (Log-likelihood $X^2_7 = 6.70$, $P = 0.461$) but did diverge between salinity treatments ($F_{1, 489} = 20.08$, $P < 0.001$). Low salinity embryos initially developed faster than those in high salinity (Fig. 2A). For example, on day 5, low salinity embryos reached an estimated stage of 19.47 versus 18.30 for high salinity embryos. Development converged temporarily at stage 23.5 on day 11 before beginning to diverge again as embryos entered larval stages on day 16. By this time, low salinity larvae reached an estimated stage of 25.47 compared to 25.28 for high salinity larvae. We observed six different types of malformations during embryonic development (Supplementary Table 1). Malformations were found only in the high salinity treatment, where 100% of individuals developed some form of malformation. The model selected for inferring malformation presence contained additive main effects with random effects for individual, family, and population. Presence of malformations increased as a second-order polynomial function of developmental stage ($X^2_1 = 549.0$, $P < 0.001$) while population type had no effect ($X^2_1 = 0.200$, $P = 0.655$). The intercept of the model was −26.58 (95% CI = −30.37 to −23.10) and thus was significantly different than zero. Across both population types, 89% of all malformations that were initially detected resolved (i.e., were no longer detectable) by the time embryos reached feeding stage (Gosner stage 25). The greatest presence of malformation was estimated to occur at stage 18.1 (Fig. 2B). Survival was high across the experimental period and did not differ with respect to salinity (high salinity hazard ratio = 2.36, 95% CI = 0.61–9.13) or population type (woodland population type hazard ratio = 0.43, 95% CI = 0.11–1.67). Across all experimental units, 95.4 % of embryos survived to feeding stage. Throughout the experiment, water temperature averaged 12.8 °C

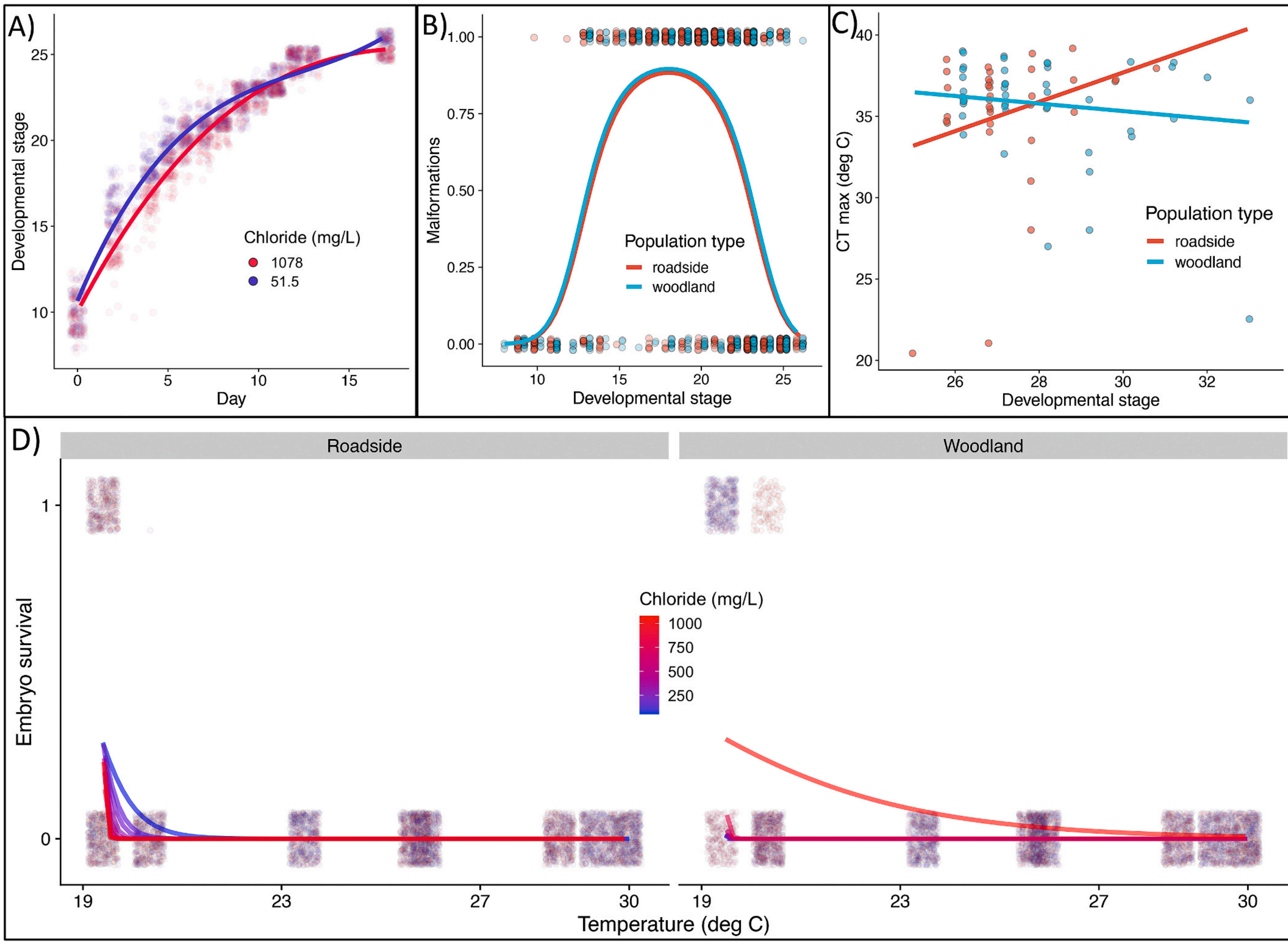

Fig. 2. Salinity and road proximity effects on developmental malformations, developmental rate, thermal physiology, and survival. (A) Developmental rate diverged between low and high salinity treatments, with developmental delays occurring in high salinity until rates converged midway through hatchling stages (i.e., stages 20–25). Lines represent predicted values from a second order linear model. (B) Embryos raised in elevated salinity developed malformations. The proportion of malformations peaked at stage 18, but malformations resolved before feeding stage in 89% of individuals. Lines represent predicted values from a second order linear model are shown. Malformations did not differ between roadside and woodland populations. (C) Among wild-collected tadpoles, critical thermal maxima varied across the interaction of developmental stage X population type. CT max increased with developmental stage for roadside populations but decreased for woodland populations. Lines show predicted values from a generalized linear model. D) Survival across 36 treatments of salt × temperature interactions is shown as a binary response, with each (jittered) point corresponding to one embryo. Roadside embryos are shown in the left panel with woodland embryos in the right. Fitted values from the generalized linear model are plotted as lines. Point and line color gradient corresponds to chloride concentration.

(±6.05 SD).

3.2. *Critical thermal maximum (CT max) in wild tadpoles*

For assessing CT max in wild-collected tadpoles, AIC selection supported the model containing random effects for population and experiment round (delta AIC = 3.34). CT max varied as a function of the interaction between developmental stage and population type (Log-likelihood $X^2_1 = 5.34$, $P = 0.021$). For each stage of development, CT max increased by 0.90 °C in roadside populations but decreased by 0.23 °C in woodland populations (Fig. 2C).

3.3. *Interacting effects of salinity and temperature on survival*

In the context of the multiple-stressors (i.e., temperature and salinity) exposure experiment, embryos had an average starting Gosner stage of 10.0 (±1.2 SD). Gosner stage did not differ as a function of population type ($F_{1, 8} = 0$, $P = 1$). Embryonic survival varied across the three-way interaction of salinity, temperature, and population type (Fig. 2D), as evidenced by significant likelihood ratio tests produced between the three-way model and each pairwise comparison with all other possible models varying in additive and interacting effects (all $P < 0.001$; Supplementary Table 2). Temperature had a strong, negative effect on survival regardless of salinity, and this effect was more severe for roadside than woodland populations. Woodland population survival declined from 60.0% at 19.4 °C to 14.6% at 20.3 °C to 0.0% in all warmer treatments. Roadside population survival declined from 42.4% at 19.4 °C to 0.0% at 20.3 °C and all warmer treatments.

3.4. *Population and family-level components of salinity tolerance*

3.4.1. *Overall survival and days to mortality*

For overall survival, the model containing random effects for population and family nested within population was preferred over the other two models (delta AIC = 40.3) and therefore selected for inference. Among main effects, survival differed across population type ($X^2_1 = 4.89$, $P = 0.027$) and salinity ($X^2_1 = 209.7$, $P < 0.001$), but not across their interaction ($X^2_1 = 0.079$, $P = 0.778$). For woodland populations, model-estimated survival values declined from 0.540 in 51.5 mg/L Cl-to 0.069 in 1078 mg/L Cl-, whereas for roadside populations, survival estimates declined from 0.243 to 0.020 (Fig. 3A). Among candidate models for days to mortality, the model containing random effects for population and family nested within population was preferred over the other two models (delta AIC = 73.3) and therefore selected for inference. Days to mortality differed across the interaction of population type and salinity ($F_{1,\ 699.7} = 13.57$, $P < 0.001$). Individuals from roadside ponds survived an average of 33.2 days (95% CI = 28.8 to 37.6) in 51.5 mg/L Cl-versus 19.6 days (95% CI = 15.2 to 24.0) in 1078 mg/L CL-, while individuals from woodland populations survived an average of 43.5 days (95% CI = 39.1 to 47.8) in 51.5 mg/L Cl-versus 24.8 (95% CL = 39.1 to 47.8) in 1078 mg/L CL- (Fig. 3B).

3.4.2. Family-level variance and repeatability of days to mortality

V_F varied across population type and salinity treatment combinations, with notable decreases from low to high salinity for both woodland and roadside populations (Fig. 3C; Supplementary Table 3). For roadside populations, V_F was 10x higher when individuals were reared under low than under high salinity conditions (posterior difference between low and high salinity: mean = 3.702, mode = 3.36, 95% CI = 2.541 to 5.092). For woodland populations, V_F was 3.9x higher in the low salinity treatment (posterior difference between low and high salinity: mean = 3.17, mode = 3.138, 95% CI = 1.412 to 5.162). There was no difference in V_F between roadside and woodland populations under low salinity conditions (posterior difference between roadside and woodland populations: mean = −0.159, mode = 0.511, 95% CI = −2.605 to 1.994), but woodland populations presented higher V_F under high salinity (posterior difference between roadside and woodland populations: mean = −0.692, mode = −0.647, 95% CI = −0.998 to −0.417). Similarly, R did not differ between population types under the low salinity treatment (posterior difference between roadside and woodland populations: mean = −0.003, mode = 0.011, 95% CI = −0.087 to 0.079). However, R decreased for both populations under high salinity in comparison to low salinity treatment (posterior difference between low and high salinity treatment for woodland populations: mean = 0.282, mode = 0.285, 95% CI = 0.192 to 0.37; for roadside populations: mean = 0.512, mode = 0.503, 95% CI = 0.443 to 0.583). Moreover, woodland populations presented higher R under high salinity conditions (Supplementary Table 4; posterior difference between roadside and woodland populations: mean = −0.232, mode = −0.239, 95% CI = −0.309 to −0.146).

3.4.3. Osmolality

For assessing whole body osmolality in larvae from the family-based salinity exposure experiment, AIC selection indicated the strongest

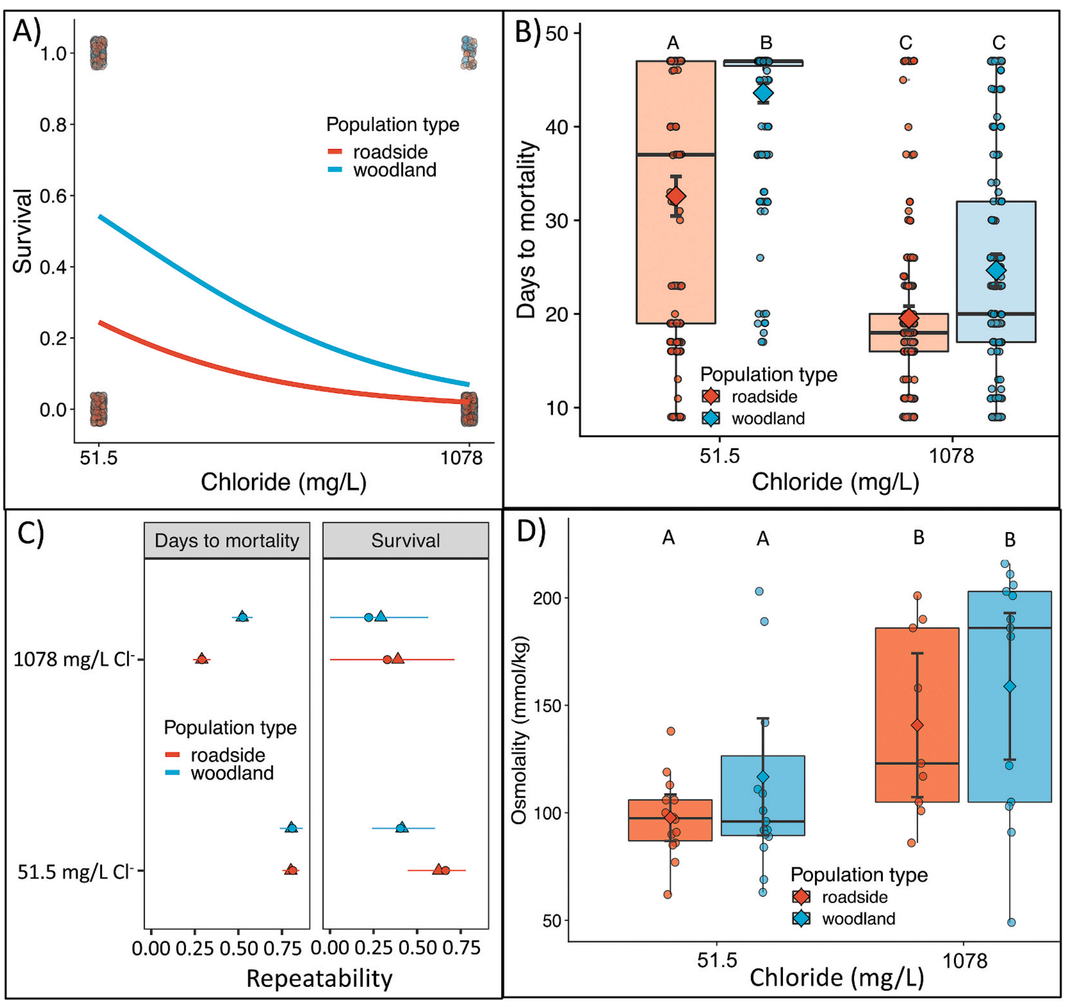

Fig. 3. Salinity and population effects on survival and repeatability. (A) Survival and (B) days to mortality are shown following chronic exposure to elevated salinity. Points are jittered to reduce overlap. Survival is shown as a binary outcome with fitted lines corresponding to back-transformed probit model estimates. Days to mortality is shown for each individual embryo and is grouped by population type. Boxplots are overlaid. Diamonds and error bars represent estimated marginal means and corresponding 95% confidence intervals. Contrasts between estimated marginal means are indicated by text symbols above each group. (C) Repeatability estimates for days to mortality and survival in each of the two salinity treatments are shown as means (triangles) and modes (circles) with lines corresponding to 95% credible intervals. (D) Larvae raised in high salinity maintained higher body osmolality than larvae raised in low salinity conditions.

support for the model with only main effects and no interactions (Supplementary Table 5). This model was used for inference. Osmolality varied in response to salt ($F_{1,\ 42.2} = 12.92$, $P < 0.001$; Fig. 3D) but not developmental stage ($F_{1,\ 45.5} = 0.16$, $P = 0.690$) or population type ($F_{1,\ 8.06} = 0.76$, $P = 0.410$). In a model with only salt for a main effect, osmolality was estimated at 149.1 mmol/kg (95% CI = 125.2 to 172.8) in high salinity versus 107.3 mmol/kg (95% CI = 64.9 to 149.2) in low salinity.

3.5. Environmental variation in natural ponds

For specific conductivity, the model that included one random effect for pond was preferred over the model with random effects for both pond and survey date (delta AIC = 3.69). Across natural breeding ponds, specific conductivity varied as a function of pond type ($F_{1,\ 14.6} = 57.9$, $P < 0.001$). Roadside pond conductivity, averaged across all measurements, was 506 (±279 SD) μS/cm compared to 52.9 (±20.5 SD) μS/cm in woodland ponds (Fig. 4A). For dissolved oxygen, the less parameterized model containing one random effect for pond was preferred (delta AIC = 1.54). There was marginal support for lower dissolved oxygen in roadside ponds ($F_{1,\ 9.90} = 5.18$, $P = 0.089$), with values across all measurements averaging 3.68 mg/L (±2.15 SD) compared to 4.36 mg/L (±1.39 SD) in woodland ponds. In the continuous analysis of dissolved oxygen as a function of conductivity, the model containing random effects for both pond and survey date was preferred over the model with only pond as a random effect (delta AIC = 2.03). Dissolved oxygen declined with increasing values of the natural log of conductivity ($F_{1,\ 8.24} = 15.46$, $P = 0.004$; Fig. 4B). Temperature averaged 13.1 °C (±3.97) and did not differ between pond types ($F_{1,\ 5.97} = 0.32$, $P = 0.591$) or as a function of conductivity ($F_{1,\ 5.97} = 0.125$, $P = 0.736$).

4. Discussion

Under cool-weather conditions, elevated salinity induced malformations in all wood frog embryos. No malformations occurred in low salinity. Most malformations resolved before hatching, and salinity had no effect on embryo survival. Under relatively warmer conditions, and across a range of elevated temperature, salinity was highly toxic, causing high mortality in embryos and in early-stage hatchlings. Roadside populations were more sensitive to this temperature-dependent salt toxicity. However, larvae collected directly from roadside ponds showed increased tolerance to temperature (CT max) compared to woodland larvae. Salt toxicity measured in terms of survival and days to mortality varied across population types, with days to mortality presenting larger reductions in and, therefore, lower estimates of family-level variance and repeatability for roadside populations under high salinity in comparison to the lower salinity environment. Taken together, this suite of results suggests that salinity has important negative consequences for wood frog populations, and that temperature plays an important role in salinity tolerance and its evolution, potentially mediating tradeoffs.

The relative sensitivity of roadside populations to temperature and salt indicates that population of origin interacts with multiple stressors, suggesting that natural selection operating in the natal ponds of these individuals might involve tradeoffs between salinity and temperature. We found no evidence that water temperature differed systematically between roadside and woodland origin ponds. Thus, if we assume that thermal selection is roughly equivalent across population types, our results suggest that exposure to pollution compromises thermal tolerance. This outcome could be the result of a tradeoff, for instance, if adaptation to roadside environments comes at the cost of reduced tolerance to other stressors, including increased temperature. Taken at face value, this increased sensitivity would suggest that polluted populations face elevated mortality risk from warming pond temperatures caused by climate change. However, in the wild, larvae from polluted ponds showed increased not decreased thermal tolerance (CT max). The contrast between these sets of outcomes suggests that thermal tolerance might be induced in the wild but not in our experimental venue. Indeed, CT max increased with increasing developmental stage, suggesting a developmental mechanism. Similarly, embryonic stages might be especially sensitive to thermal stress, causing high mortality in our experiment before thermal tolerance traits could develop in larvae. Alternatively, the functional benefit of increased thermal tolerance evidenced by elevated CT max might manifest at temperatures lower than those used in our experiment. Indeed, one limitation of our experiment was that the lowest temperature treatments exceeded temperatures typically found in ponds during embryonic and early larval development. Regardless of mechanism, increased CT max in roadside larvae remains difficult to explain in the absence of evidence of increased pond temperature. Thus, future studies should examine temperature ranges that better capture the values found in natural ponds.

The stark contrast in survival found between the outdoor, cool-temperature experiment and the indoor salinity X temperature

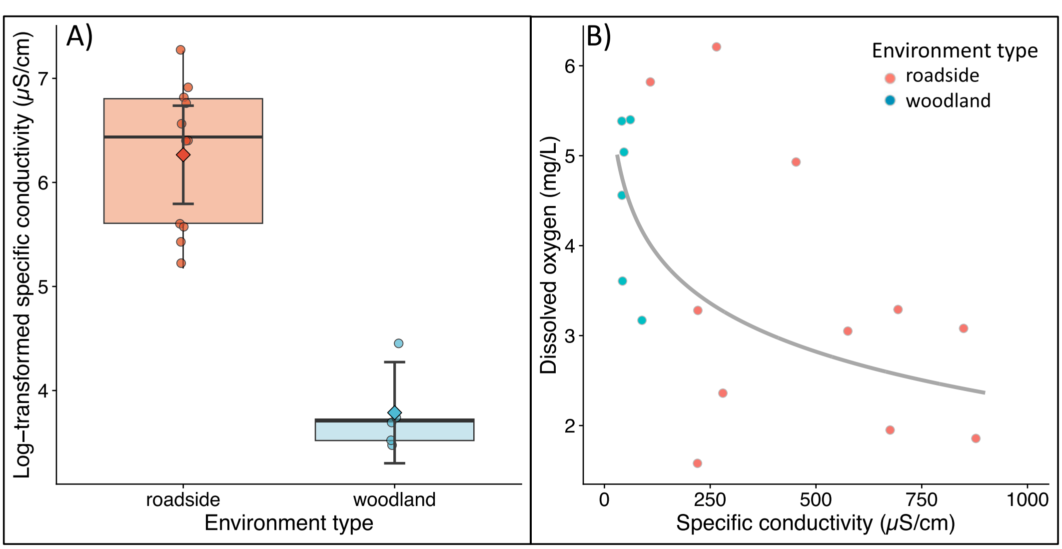

Fig. 4. Environmental differences between roadside and woodland ponds. (A) Roadside ponds had elevated levels of (log-transformed) specific conductivity, a close proxy for road salt. Points represent average pond values; diamonds indicate mean values with 95% confidence limits. (B) Dissolved oxygen declined with increasing (log-transformed) specific conductivity. Points represent average pond values; fitted line represents the main effect from the mixed model.

experiment was surprising. In addition to temperature effects, experimental venue might have contributed to this difference. Namely, embryos in the outdoor experiment were housed individually within well plates, whereas embryos indoors were raised in 5-embryo clusters, with 25 clusters grouped into a single 5.5 L container. Stocking density per se was not highly dissimilar (ca. 31 vs 59 embryos per liter). This raises the intriguing possibility that proximity to other embryos and/or other-pond embryos might influence toxicity, if for example embryonic osmoregulation is compromised by eggs being in contact with each other, or if responses to stress have negative effects on adjacent individuals (e.g., via density-dependent diseases). Thus, being reared in isolation might to some extent buffer the negative effects of salinity stress. Interestingly, even a slight increase in Cl-concentration from 51.5 to 120 mg/L Cl-caused increased mortality at the lowest of the warmer temperatures. Notably, 120 mg/L Cl-matches the chronic exposure criterion for aquatic life set by the Council of Ministries of the Environment in Canada (CCME., 2011) and is about half the 230 mg/L Cl-value set by the Environmental Protection Agency in the U.S. (EPA., 1988), suggesting that these criteria might not be widely protective (Hintz et al., 2022), particularly at warmer temperatures.

Despite differences in survival and days to mortality, roadside and woodland population types showed similar changes in internal osmolality in response to elevated salinity. Therefore, it appears that osmoregulatory processes in both population types maintain equivalent levels of relative osmotic homeostasis. This outcome indicates that internal osmolality might not underpin survival differences between population types. However, the process of maintaining internal osmolality levels that match those of woodland larvae could be more costly for roadside populations (contributing to increased mortality) and could potentially be linked to tradeoffs with traits that increase components of fitness in other contexts or at later life history stages. For instance, roadside populations might invest energy into physiological processes that produce adaptive outcomes in roadside environments, such as ArH pathways that facilitate detoxification of organic contaminants found in polluted habitats (Vogel et al., 2020). Conceivably, the energy required to maintain osmotic balance in high salinity conditions might limit energy allocated to coping with other stressors, and in the absence of those other stressors, roadside populations have relatively lower survival compared to woodland populations. Alternatively, equivalent osmolality might be a methodological artifact. By measuring osmolality only in surviving larvae, we might have missed a correlation between osmolality and survival and/or an interaction between salinity and population type. For instance, roadside individuals that died prior to the end of the experiment could have had relatively high osmolality (the so-called 'missing fraction' problem). Another interesting aspect of this experiment was the resolution of embryonic malformations prior to hatching. Prompted by these results, we inspected a time-lapse video captured anecdotally as a demonstration of salinity effects on embryonic development. Images from this video (Supplementary Fig. 2) show that the vitelline membrane in high salinity fails to enlarge—presumably because of reduced osmotic pressure in the hypertonic external environment (Beattie, 1980)–and appears to constrain the embryo from straightening. This spatial effect would appear to explain the temporary nature of the malformations we observed, since once the embryos hatch, they could fully extend their body. It also is possible that embryonic development is particularly robust to developmental disturbance. Indeed, other studies of chronic salt exposure have found lasting axial malformations that manifest in larvae after hatching (Sanzo and Hecnar, 2006), and which occur more often in roadside than woodland populations (Brady, 2013). However, it remains unknown whether the incipient but resolved malformations detected here negatively affect fitness later in life. Thus, the resolution of these malformations does not necessarily mean that they are cost free.

In low salinity, variation in survival probability was partially due to differences between families, particularly so for roadside populations. However, in high salinity environments, variation in survival could not be associated to differences between families for either population type, as taken from the low family-level variances and repeatabilities. Repeatabilities of family-level variance indicates that traits are more similar among siblings than across individuals from different families. Similarity among siblings suggests a shared mechanism for survival, resulting from either shared genetic or parental environmental effects. Therefore, our results contrast a previous finding of substantial variances and GxE at the family-level for survival under salinity stress, as well as positive paternal effects on the survival of tadpoles (Brady and Goedert, 2017). Lower sample sizes may have therefore prevented us from estimating such variances in the current study, as evidenced by the large credible intervals around our estimates. Higher salt concentrations used in the current study may also have exceeded the range in which individuals can vary, as found under lower salinity. Indeed, high salinity eroded large family-level variances for days to mortality found in low salinity, particularly for the roadside populations. The ability to tolerate salinity stress for longer durations may be a key factor in allowing these populations to adapt to environments exposed to salt runoff, particularly since early developmental stages are crucial in limiting survival for roadside populations (Forgione and Brady, 2022). Here, the decrease in family-level variance could also be indicative of erosion of adaptive transgenerational parental effects, wherein parents favorably alter the phenotype of the offspring through means beyond genetic inheritance, such as egg provisioning of yolk, hormones or mRNA, or via epigenetic inheritance (Mousseau and Fox, 1998; Rando, 2012). Considering previous reports of paternal effects on embryo survival (Brady and Goedert, 2017), such effects may play a significant role in salinity tolerance for wood frogs, at least under lower levels of salinity stress. Therefore, in the wild, ponds with high salinity (as were those studied here) might constrain the capacity for populations to adapt via viability selection on survival or days to mortality by decreasing the expression of the underlying genetic variation or by depleting adaptive parental effects that may allow populations to persist even in the absence of adaptive genetic variation. Of course, high salinity is just one of many contaminants typical of roadside ponds, joining a bevy of pollutants like aromatic hydrocarbons, heavy metals, and litter, and these contaminants might alter other aspects of water quality. For instance, here we found for the first time that saltier ponds had lower dissolved oxygen concentrations. Though the mechanism remains unclear, this relative reduction in oxygen availability might further stress wild populations during embryonic and larval stages of development. Further, reduced oxygen availability in salinized freshwater ponds might generate compound effects on salinity tolerance if, for instance, lower oxygen reduces gill development or osmoregulatory function, much like the impact salinity is thought to have on wood frog gills (Szeligowski et al., 2022). Ultimately, oxygen limitation in freshwater ponds might be worsened by rising surface water temperatures from climate change, predicted to reduce the amount of oxygen dissolved in freshwater (Ficke et al., 2007), highlighting the need to consider oxygen availability as a potentially important stressor in salinized freshwater habitats.

A growing body of literature suggests that some freshwater organisms have the capacity to rapidly adapt to some degree of salinization (Albecker and McCoy, 2017; Coldsnow et al., 2017; Hintz et al., 2019; Arnott et al., 2023). Moreover, stressful environments have been reported to uncover standing additive genetic variance, possibly facilitating adaptive responses to a new environmental stressor (Badyaev, 2005; Rowiński and Rogell, 2017). However, our results suggest that wood frogs facing increasing salinization of their environments may instead present reduced additive genetic variance, causing maladaptation to persist due to reduced evolvability. Alternatively, similar or even higher additive genetic variance is being over-shadowed by a reduction in transgenerational parental variance under the high salinity environment. In that case, maladaptation patterns may be enhanced by a reduced tolerance to the stressful condition, which could ultimately affect adaptability for these populations (Badyaev, 2005; Derry et al., 2019). Fully elucidating how our reported decrease in family-level variance impacts the ability of wood frogs to adapt and persist in salinized habitats therefore requires additional studies aimed at further partitioning family-level variance into its multiple components. Finally, additional factors may also interact with a potential reduction in genetic variance due to stress. For instance, the philopatric nature of wood frogs may be further detrimental for these populations, as it reduces the opportunity for evolutionary rescue via introgression of beneficial genetic variants originating in other populations (Bell, 2017). Lack of gene flow may additionally result in inbreeding and inbreeding depression, which can even be amplified whenever genetic effects change across environmental conditions (e.g., Cheptou and Donohue, 2011; Sandner et al., 2021). Conceivably, roadside populations might be more isolated from other populations since roads can reduce gene flow for amphibians (Garcia-Gonzalez et al., 2012; Fusco et al., 2021), and thus higher rates of inbreeding might be expected.

Understanding the potential for population-level adaptation and the scope of tolerance across freshwater populations will be vitally important to guiding pollution policy and management (Arnott et al., 2023). Yet despite the ubiquity of multiple stressors found in freshwater habitats, we are only beginning to understand how their interactions can shape populations such as through the evolution of cross tolerance or costs to tolerance (Van de Maele et al., 2021; Zhao et al., 2023). Thus, our knowledge of interacting stressor effects in wild populations remains poorly understood. A key limitation of our study is the gap in temperature between the outdoor exposure experiment and indoor temperature X salinity experiment. In the former, temperature averaged 12.8 °C; in the latter, the lowest temperature was 19.4 °C, which is on the higher side of what wood frog embryos experience in nature within our study region. A key strength, however, is that our study is among the first to consider salinity and temperature stress in tandem. Our study additionally provides important insights into the interactions not only between these two stressors, but also among population origin, highlighting the complex responses we can expect in natural populations. Future multi-stressor investigations that include lower temperature values should be profitable.

Countless anthropogenic stressors now confront wild populations with a seemingly limitless suite of novel conditions acting as agents of selection that vary in timing and intensity, and can interact with life history, genetic variation, and other evolutionary forces to mediate fitness and population demographic outcomes. For wood frog populations, temperature appears to be an important stressor that can interact with elevated salinity to exacerbate maladaptive survival patterns in larvae. Understanding the role of temperature in mediating salt toxicity should be a priority as these two stressors become increasingly common across the landscape due to human activities and climate change.

CRediT authorship contribution statement

Lauren M. Conner: Conceptualization, Data curation, Investigation, Methodology, Visualization, Writing – original draft, Writing – review & editing. **Debora Goedert:** Conceptualization, Formal analysis, Methodology, Resources, Visualization, Writing – original draft, Writing – review & editing. **Sarah W. Fitzpatrick:** Conceptualization, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Writing – original draft, Writing – review & editing. **Amber Fearnley:** Data curation, Investigation. **Emma L. Gallagher:** Data curation, Investigation. **Jessica D. Peterman:** Data curation, Investigation. **Mia E. Forgione:** Data curation, Investigation. **Sophia Kokosinska:** Data curation, Investigation. **Malik Hamilton:** Data curation, Investigation. **Lydia A. Masala:** Data curation, Investigation. **Neil Merola:** Data curation, Investigation. **Hennesy Rico:** Data curation, Investigation. **Eman Samma:** Data curation, Investigation. **Steven P. Brady:** Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Writing – review & editing, Resources, Supervision, Validation, Visualization, Writing – original draft.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.chemosphere.2024.141978.

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