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Effects of road salt, egg predation and alterations to the egg-mass jelly layers on the embryos of spotted salamander *Ambystoma maculatum*

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Road de-icing salt has been used to make winter driving safer. However, its eventual drainage into adjacent water bodies has caused detrimental effects on organisms in those aquatic communities, including amphibians. We used the spotted salamander *Ambystoma maculatum* as a model species and examined whether there would be additive or interactive effects of road salt, egg predation from wood frog *Lithobates sylvaticus* tadpoles, and alteration to egg-mass jelly layers on the hatching rate, incubation period, snout-vent length (SVL) and developmental stage of hatchlings. We found neither main nor interactive effects of salt on the measured traits, which contradicts some of the previous studies. The other body of previous studies, however, showed observational and experimental evidence that amphibian populations became locally adapted to saline water. Given the roadside origin of our study animals, our negative results provide insight into the ubiquity of local adaptation to saline water among amphibian populations. We also found that egg predation and the presence of egg-mass jelly layers negatively affected the incubation period, SVL, or development in the predicted manners based on the O_2 demand-supply balance within egg masses. These responses might be altered by dissolved salt if populations naive to freshwater salinisation were exposed to saline water.

Keywords: aquatic pollution, community ecology, conservation, larval amphibians, *Lithobates sylvaticus*

INTRODUCTION

Despite increasing attention and conservation efforts in recent years, approximately 40 percent of amphibian species have become threatened with extinction due to various direct and indirect human causes, and pollution is one of the major causes along with habitat destruction/fragmentation, invasive species, diseases, light and noise pollution and climate changes (Wake, 1991; Forman & Alexander, 1998; Collins et al., 2009; Lötters et al., 2009; Jones et al., 2017; Dutta, 2018; Lind et al., 2018; Hintz & Relyea, 2019; Fisher & Garner, 2020). Freshwater aquatic habitats adjacent to roads are susceptible to anthropogenic pollution since they trap chemicals from road runoff (Mungur et al., 1995; Dobson et al., 1997; Turtle, 2000; Hayes et al., 2002; Godwin et al., 2003; Collins & Russell, 2009; Denoël et al., 2010; Stoler et al., 2017; Schuler & Relyea, 2018; Schuler et al., 2019). Pollutants entering the water affects the salinity, turbidity and dissolved oxygen levels (Forman & Alexander, 1998; Schuler & Relyea, 2018), which can alter food webs (Jones et al., 2017; Lind et al., 2018; Schuler & Relyea, 2018) and reduce the biodiversity of aquatic communities (Dobson et al., 1997; Forman & Alexander, 1998; Collins & Russell, 2009; Denoël et al., 2010; Stoler et al., 2017; Schuler & Relyea, 2018).

Sodium chloride (NaCl) is one of the pollutants that enter the roadside freshwater ecosystems because it is commonly used as road de-icing salt to make winter travels safer. In the United States, for example, studies reported increases in concentrations of sodium and chloride in roadside aquatic habitats over the past decades (Lagerwerff & Specht, 1970; Godwin et al., 2003; Brady, 2012). Pond-breeding amphibians are the members of aquatic communities facing the detrimental effects of freshwater salinisation (Karraker et al., 2008; Collins & Russell, 2009; Karraker & Gibbs, 2011; Jones et al., 2017; Schuler & Relyea, 2018; Ocampo et al., 2023). Pond-breeding amphibians are vulnerable to aquatic pollutants because they rely on aquatic habitats to complete their life cycle, have semi-permeable skin (Denoël et al., 2010) and tend to be sedentary (Sanzo & Hecnar, 2006). Furthermore, road salt together with other biotic (e.g. predators and competitors) (Karraker & Ruthig, 2009; Jones et al., 2017) and abiotic stressors (e.g. metals, pesticides and fertilisers) (Stoler et al., 2017; Schuler & Relyea, 2018; Lewis et al., 2021) threaten amphibians, often through complex pathways.

The spotted salamander *Ambystoma maculatum* is one of the pond-breeding amphibians commonly found in roadside pools in eastern North America. Acute toxicity

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tests revealed larvae of *A. maculatum* were one of the most sensitive pond-breeding amphibians to chloride in Nova Scotia, Canada (Collins & Russell, 2009). Freshwater salinisation also decreases the survivorship of the embryos (Turtle, 2000; Karraker et al., 2008). Karraker & Gibbs (2011) also found high chloride concentrations dehydrated the thick outer jelly layers surrounding egg capsules of *A. maculatum* and permanently disrupted their osmoregulation, resulting in delayed hatching and reduced hatching success of *A. maculatum* embryos (Karraker & Gibbs, 2011). The jelly layers provide essential protection for developing *A. maculatum* embryos against predators (Ward & Sexton, 1981). However, the wood frog *Lithobates sylvaticus* tadpoles still can significantly lower the survivorship of *A. maculatum* embryos (Petranka et al., 1998; Holbrook & Petranka, 2004). Furthermore, intact jelly layers can reduce the survivorship and developmental rate of the salamander embryos because the thick outer jelly layers interfere with O_2 diffusion throughout the egg mass (Pinder & Friet, 1994; Hale et al., 2017). Therefore, the jelly layers, which protect embryos from predation under normal circumstances, may impose negative impacts on the embryos when the water becomes salinised, and these hypothesised negative effects of salinisation may further be altered by the presence of egg predators that remove the jelly layers by eating them before reaching the embryos inside.

Here, we investigated the effects of road salt, egg predation and manipulation of egg-mass jelly layers on the hatching success, incubation period, developmental stage at hatching and hatchling body size of the spotted salamander *A. maculatum*. In order to investigate the main and interactive effects of these three factors (i.e. salinisation, predation and jelly layers), we conducted a laboratory experiment, in which we created eight treatments by fully crossing the three variables, each of which had two levels: presence/absence of road salt, presence/absence of tadpole predation and presence/absence of jelly layers.

MATERIALS & METHODS

Field procedure

Egg masses of the spotted salamander *A. maculatum* and wood frog *L. sylvaticus* were collected from four vernal pools in Union County, Pennsylvania, USA on 10 April 2018. The vernal pools were monitored for two weeks before the collection day so we knew the age of each egg mass. The first major breeding and oviposition of *L. sylvaticus* occurred between 27 and 29 March 2018, whereas the first major oviposition event of *A. maculatum* occurred on the night of 9 April 2018. *Ambystoma maculatum* deposits two major types of egg masses, clear and white (Ruth et al., 1993), however, only clear egg masses were collected so that the number of embryos inside of each egg mass was visibly assessed before the experiment. Thirty-two salamander egg masses were used for the experiment. Several egg masses of *L. sylvaticus* that were laid on the same day were also collected, from which approximately 500 embryos were isolated for the experiment.

Experimental setup

The experiment consisted of eight treatments based on a factorial design of three main factors: presence/absence of road salt, presence/absence of *L. sylvaticus* tadpoles (predators) and presence/absence of the jelly layers of the salamander egg masses. Each treatment was replicated four times, which resulted in 32 experimental containers. Each contained a single egg mass of *A. maculatum*. In order to calculate a hatching rate, we counted the number of salamander embryos in each egg mass by gently pressing them with a transparent glass sheet and taking photos before placing them into each container (a mean number of embryos per mass ± SE = 124.4 ± 3.6). All containers (container size: 28 cm[L] x 15 cm[W] x 11 cm[H]) were randomly assigned to the eight treatments and arranged in a single incubator under a 14-hour light/10-hour dark cycle with a temperature originally set at 12.7 °C. The National Weather Service Forecast website was used to calculate the historical monthly average temperatures of April and May in this area from 2000 to 2018. Based on the monthly temperature difference between April and May, we increased the incubator temperature by 1.5 °C every Friday during the experiment in order to reflect the general weather patterns of the area.

The treatments with the presence of road salt had a salt (NaCl) concentration of 1000 mg/L (equivalent to 607 mg/L chloride), which is within the range of the ecologically-relevant salt concentration found in vernal pools adjacent to roads (Environment Canada & Santé Canada, 2001; Karraker et al., 2008). Based on the findings of the previous study (Karraker et al., 2008), the salt concentration in our experiment was expected to cause a 40–50% reduction in embryonic survivorship. The treatments that were not assigned with the presence of road salt received aged water. All containers were filled with aged tap water or saltwater up to 7 cm in depth.

Different numbers of *L. sylvaticus* tadpoles were added to the tadpole-treatment containers based on a 10:8 ratio of the number of *A. maculatum* embryos and the number of *L. sylvaticus* tadpoles, which was within the range of the field densities (Takahashi, personal observation). When an egg mass was assigned to the non-jelly layer treatments, the thick, outer, gelatinous layers surrounding the egg capsules were carefully removed, and all of the individual egg capsules were placed in the container filled with aged tap water or salt water. Additionally, we fed rabbit food in all containers with *L. sylvaticus* tadpoles whose hatching occurred between 16 and 24 April 2018. The experiment was started on 25 April 2018 when all of the salamander eggs and tadpoles were placed into their corresponding treatment containers. We fed tadpoles and changed 50% of the water for all containers weekly throughout the experiment.

Data collection

All 32 containers were monitored daily for *A. maculatum* hatchlings between 25 April 2018 and 28 May 2018 when the last hatchling appeared. The number of *A. maculatum* hatchlings from each of the 32 containers was recorded. Collected hatchlings were immediately euthanised with

Table 1. Results of ANOVAs testing the effects of salt, tadpole predation and jelly-layer manipulation on hatching success, incubation period, SVL and developmental stage for *Ambystoma maculatum* embryos. Bold numbers with asterisks indicate statistically significant p-values.

	Hatching success			**Incubation period**			**Snout-vent length (SVL)**			**Developmental stage**		
	df	F-value	p-value	df	F-value	p-value	df	F-value	p-value	df	F-value	p-value
Salt	1,23	1.412	0.249	1,19	12.899	0.329	1,19	5.082	0.970	1,19	2.881	0.305
Tadpole	1,23	2.839	0.108	1,19	0.175	0.68	1,19	24.744	**0.008***	1,19	31.445	**0.0021***
Jelly	1,23	2.034	0.17	1,19	1.006	**0.002***	1,19	0.001	**0.036***	1,19	1.11	0.106
Salt x tadpole	1,23	0.098	0.758	1,19	1.945	0.067	1,19	0.445	0.886	1,19	4.567	0.211
Salt x jelly	1,23	0.018	0.895	1,19	0.075	0.787	1,19	1.053	0.318	1,19	0.041	0.841
Tadpole x jelly	1,23	1.778	0.198	1,19	3.763	0.179	1,19	0.021	0.513	1,19	1.677	**0.046***
Salt x tadpole x jelly	1,23	1.936	0.18	1,19	1.231	0.281	1,19	0.51	0.484	1,19	0.022	0.885

an overdose of MS-222 by immersion and preserved for the determination of developmental stages and body size measurements. The Harrison (1969) standardised salamander staging chart was used in order to identify the developmental stages of the *A. maculatum* embryos. The presence/absence of a leg bud, body colouration, length of balancer, complexity/branching of the gills, and presence/absence of a hind leg bud were used to determine their developmental stages (Harrison, 1969). We used a LEICA EZ4HD dissecting microscope with a built-in digital camera to examine the developmental stages as well as to take pictures of each preserved specimen for body size measurements. We used ImageJ (Schneider et al., 2012) for measuring the total body length, SVL and tail length of each individual hatchling. Over the course of the experiment, the signs of water mould infection were observed in five containers, which were removed from the data analyses. Those containers were two of the no-salt, yes-predators and yes-layers containers; one of the yes-salt, yes-predators and no-layers containers; and two of the yes-salt, yes-predators and yes-layers containers. Therefore, the following data analyses were done using the data from the remaining 27 containers.

Data analysis

The number of hatchlings per container ranged from zero to over 100. If there were under 20 total hatchlings in a container, all of the 20 hatchlings were measured and used for the growth and development analyses. When the number of hatchlings in a container was greater than 20 and less than 100, 20 individuals were randomly selected for the growth and development analyses. Finally, when the number of hatchlings in a container was greater than 100, 20% of the hatchlings (i..e more than 20 individuals) were randomly selected for the growth and development analyses. In total, 2,300 hatchlings were collected, of which 774 individuals were analysed for growth and development.

Three-way analyses of variance (ANOVAs) were run to evaluate the significance of both main and interactive effects of road salt, egg predators and jelly layers on the hatching success, incubation period, developmental stage and SVL of *A. maculatum* hatchlings. When the assumption of normal distribution or heterogeneity was violated, the data was log-transformed before ANOVA tests. All statistical analyses were conducted using the software R (R Core Team, 2021) with the R packages rstatix (Kassambara, 2021) and emmeans (Lenth, 2021).

RESULTS

Hatching rate

There were no significant main effects of salt, predation, jelly layers, nor any interactive effects between the independent variables on the hatching rate of *A. maculatum* embryos (Table 1). However, there was an interesting trend in the absence of predation and jelly layers; these embryos had the highest rate of hatching success compared to all other conditions, as larvae in the absence of these two variables had an average hatching rate of 0.93 ± 0.04 SE compared to 0.56 ± 0.08 SE for the rest of the treatment groups (Table 1; Fig. 1).

Incubation period

The presence of jelly layers significantly increased the incubation period of salamander embryos (P = 0.002), as the average incubation period with jelly layers was 31.9 days ± 0.47 SE, whereas the average incubation period of those without jelly layers was 28.6 days ± 0.81 SE (Table 1; Fig. 2). There were no significant main nor interactive effects of tadpoles and salt pollution on incubation periods (Table 1).

Snout-vent length (SVL)

The presence of tadpoles produced significantly smaller hatchlings (P = 0.008), as the average SVL of hatchlings in the presence of tadpoles was 7.0 mm ± 0.12 SE, whereas those in the absence of tadpoles had an average SVL of 7.2 mm ± 0.10 SE (Table 1; Fig. 3a). The jelly layers also had a significant effect on hatchling SVL (P = 0.036), as hatchlings in the absence of jelly layers had an SVL of 7.2 mm ± 0.10 SE, whereas those in the presence of jelly layers had an average SVL of 7.1 mm ± 0.11 SE (Table 1; Fig. 3b). There was no significant effect of salt, nor were there any significant interactive effects between the independent variables. It is important to note that means and standard

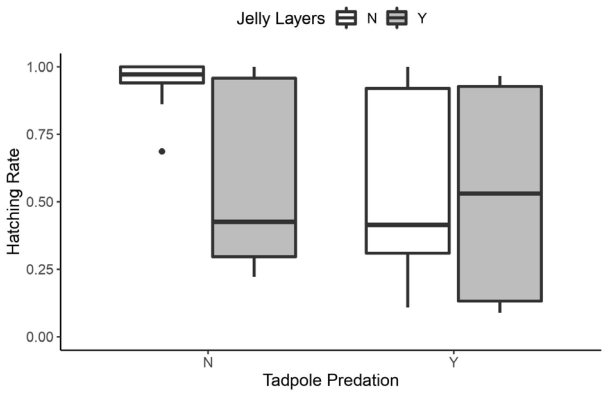

Figure 1. A boxplot showing the hatching rate (i.e. successful hatching) of *Ambystoma maculatum* embryos under four treatments with two independent variables fully crossed: the absence/presence of *Lithobates sylvaticus* tadpole predation and the absence/presence of egg-mass jelly layers.

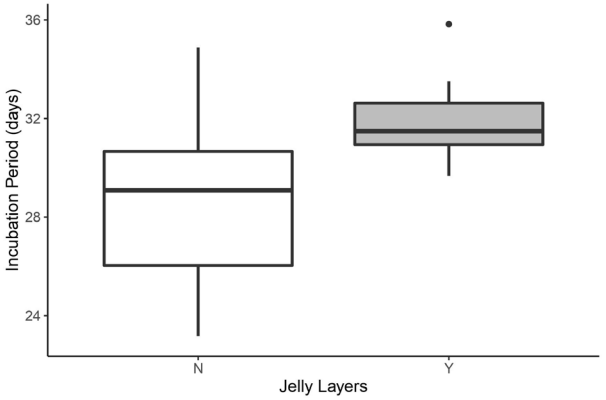

Figure 2. A boxplot showing the incubation period of *Ambystoma maculatum* embryos under two conditions, the absence or presence of egg-mass jelly layers.

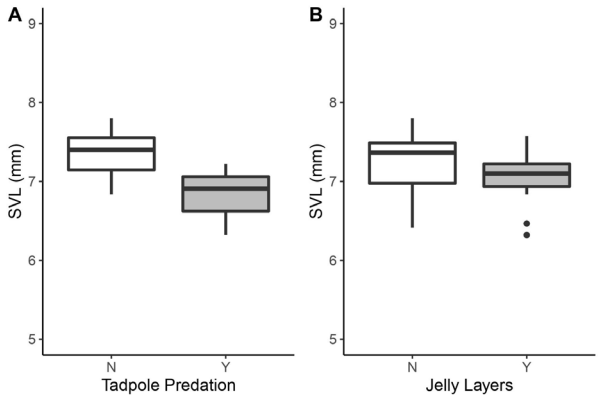

Figure 3. Boxplots showing snout-vent length (SVL) of *Ambystoma maculatum* hatchlings - **A.** under the absence and presence of *Lithobates sylvaticus* tadpole predation and **B.** under the absence and presence of egg-mass jelly layers.

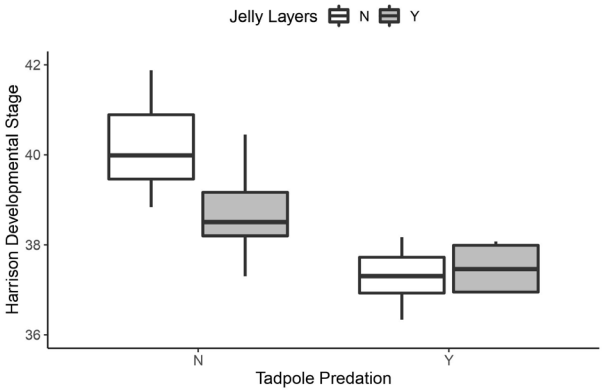

Figure 4. Boxplots Harrison developmental stage of *Ambystoma maculatum* hatchlings under four conditions with two independent variables fully crossed: the absence and presence of *Lithobates sylvaticus* tadpole predation and the absence and presence of egg-mass jelly layers.

errors reported here and used in the figures are observed values while statistical significances were based on linear models, which can create seeming disparities between p-values and reported pairwise means.

Developmental stage

The presence of egg predators alone had a significant effect on the developmental stage of salamander hatchlings (P = 0.0021), as the larvae in the presence of tadpoles had an average Harrison stage of 38.1 ± 0.28 SE, whereas those in the absence of tadpoles had an average stage of 38.9 ± 0.42 SE (Table 1; Fig. 4). The combination of egg predators and jelly layers also had a significant interactive effect on the developmental stage (P = 0.046), as the effect of jelly layers was apparent only in the absence of tadpoles, and those hatched in the absence of both tadpoles and jelly layers were the most developed at hatching with an average Harrison stage of 40.2 ± 0.36 SE (Table 1; Fig. 4).

DISCUSSION

The salt treatments with the intermediate concentration (607 mg/L chloride concentration) in this study did not significantly affect the survivorship or the growth and development of salamander embryos (N = 12 [salt treatments] vs. N = 14 [non-salt treatments]). This result was unexpected when previous studies documented the negative effects of freshwater salinisation on the survivorship of *A. maculatum* embryos (Turtle, 2000; Karraker et al., 2008). Karraker et al. (2008) further showed that (1) embryonic survival was reduced even at relatively low salinity (145 mg/L chloride concentration) though there was notable variation in survivorship from 0% to nearly 100%, and that (2) the average survivorship of embryos at relatively high salinity (945 mg/L chloride concentration) was reduced from 84% to 3%. The notable variation in embryonic survival found in the previous study (Karraker et al., 2008) suggests variation in salt

tolerance among *A. maculatum* egg masses. In fact, there have been many observations reporting amphibians from 144 species in 28 families, including *A. maculatum* (Hardy, 1952), inhabiting saline habitats (Hopkins & Brodie, 2015). Among the 144 species, the evolution of salt tolerance was evident within at least 42 species (Hopkins & Brodie, 2015). Furthermore, Brady (2012) experimentally showed that compared to woodland populations, roadside populations of *A. maculatum* survived better in saline water, by 25% on average, demonstrating local adaptation to saline conditions.

We collected *A. maculatum* egg masses for this experiment from those pools that had consistently produced a prolific number of pond-breeding amphibian eggs and larvae over the past decade. Interestingly, those vernal pools are located within 50 m of the nearby road where road salt has been used during the winter months. The conductivity measures we took in March 2017 indicated low salinity with conductivity ranging from 35–128 µS/cm (≈ 10.2–37.1 mg/L chloride concentration). Given the proximity to the road, however, it is possible that amphibian populations breeding in those vernal pools have developed local adaptation via repeated exposures to winter and spring salinisation. While our predictions about the effect of salt were rejected, our negative results add to the growing body of literature demonstrating salt tolerance of amphibians (Hopkins et al., 2016; Albecker & McCoy, 2017; 2019; Lai et al., 2019; Albecker et al., 2021).

Previous studies reported negative effects of the firm jelly layers surrounding *A. maculatum* embryos because such thick layers hinder O_2 diffusion throughout the egg mass (Pinder & Friet, 1994; Hale et al., 2017; Takahashi & Ruszala, 2023). We also found that the presence of jelly layers resulted in delayed hatching and smaller-bodied hatchlings. It has been suggested that the evolution of jelly layers has occurred in response to egg predation, and wood frog tadpoles are one of the most common egg predators (Petranka et al., 1998; Holbrook & Petranka, 2004). Therefore, the benefit of jelly layers should be manifested under a certain range of egg predation, which predicts the interactive effect between egg predators and jelly layers (Takahashi & Ruszala, 2023). We found the negative effects of egg predators on growth (i.e. body size; Fig. 3a) and development (i.e. Harrison Stage; Fig. 4) as well as the predicted pattern of the interactive effect between predators and jelly layers on their development, in which the negative effect of jelly layers was apparent only under the absence of predators (Fig. 4). However, we found no interactive effects between egg predators and jelly layers on hatching success, incubation period, and body size (Table 1). Furthermore, the hatching success of embryos without jelly layers under the predation treatment, which was predicted to be very low without the jelly-layer protection against predators, was similar to that with jelly layers under both predator treatments (Fig. 1). These results suggest that the level of egg predation created in our experiment might have been relatively low.

It is important to note that the effect of jelly layers is context-dependent and becomes detrimental when the consumption of O_2 by embryos becomes greater than the available amount of O_2. Diffusion does not provide sufficient O_2 throughout an egg mass, and thus, O_2 provision by symbiotic algae associated with jelly layers critically determines embryonic survival (Pinder & Friet, 1994). Therefore, hypoxia occurs when symbiotic algae do not provide sufficient O_2 (Pinder & Friet, 1994). In nature, these scenarios can occur in deep, cold water or shaded areas where algal photosynthesis becomes limited, or in shallow, warm water where O_2 consumption by developing embryos surpasses O_2 provision via diffusion and algal photosynthesis (Hale et al., 2017). In our experiment, the 14-hour light/10-hour dark cycle likely provided sufficient light for algal photosynthesis, but the constant daily temperature, based on the average regional temperature, without increase during daytime might have reduced photosynthesis capacity during the daytime, creating the O_2 supply-demand balance becoming slightly demand-heavy. This might be the mechanism for the presence of jelly layers resulting in longer incubation period and smaller body size at hatching.

Amphibian embryos are equipped with hatching plasticity and change hatching timing in response to environmental stimuli (Warkentin, 2011). For example, hatching timing tends to shift earlier when embryos are exposed to aquatic egg predators, often resulting in less-developed and smaller-bodied hatchlings. Such environmentally-cued hatching plasticity is adaptive because hatching earlier increases survival from egg predators (Warkentin, 1995; Touchon et al., 2011). In our experiment, the presence of egg predators did not affect the hatching timing of salamander embryos, which did not support the general trend found in Warkentin's review (2011). Furthermore, Takahashi & Ruszala (2023) found that *A. maculatum* embryos hatched earlier in response to *L. sylvaticus* tadpoles only when the embryos were not surrounded by the jelly layers. The lack of hatching plasticity in this study may be because there exists notable intraspecific variation in hatching plasticity in some species. For example, egg clutches of *A. texanum* and *A. barbouri* that hatched earlier in control conditions delayed hatching in response to egg predator flatworms *Phagocotus gracilis* while those that hatched later in control showed no response to the egg predators (Sih & Moore, 1993).

Despite the lack of accelerated hatching in response to egg predators, we found that the presence of the egg predators resulted in less-developed and smaller-bodied hatchlings. The reason for the decreased growth and development in the presence of these predators is likely to be the decreased amounts of algae found within the egg masses as a result of tadpole predation (Petranka et al., 1998). The symbiotic algae convert carbon dioxide (CO_2) produced by the salamander embryos into O_2, which the embryos exploit to survive, grow and develop. Although tadpole predation likely increased O_2 diffusion by thinning the jelly layers, our results suggest that relative to the change in O_2 diffusion capacity, the loss of the symbiotic algae imposed a greater impact on the O_2 supply-demand balance and resulted in O_2 deficient conditions.

Pollution is one of the major contributors to the global amphibian declines, and road salt has been shown

to have detrimental effects on embryos and larvae of pond-breeding amphibians (Karraker et al., 2008; Collins & Russell, 2009; Karraker & Gibbs, 2011; Jones et al., 2017; Schuler & Relyea, 2018; Ocampo et al., 2022). The other line of the literature, which has gained recent appreciation by conservation ecologists, also suggests that amphibian populations contain sufficient variation to undergo evolutionary changes in response to freshwater salinisation (Hopkins et al., 2016; Albecker & McCoy, 2017; 2019; Lai et al., 2019; Albecker et al., 2021). Contrary to our predictions, we found no main and interactive effects of salt on the survival, growth and development of *A. maculatum*. In light of local adaptation to saline habitats, negative results such as ours provide valuable insight into the ubiquity of local adaptation to saline water and thus the understanding of how global freshwater salinisation impacts amphibians. Although salt did not impose interactive effects with the two other variables (i.e. egg predators and jelly-layer manipulation), these two variables independently and interactively affected embryonic hatching, growth or development in the predicted manners based on the O_2 supply-demand balance within egg masses. These responses may be altered when embryos from naive populations of *A. maculatum* are exposed to saline water. Future studies should also consider examining the effects of salt pollution on amphibians throughout metamorphosis and sexual maturation. Exposure to saline environments during embryonic and larval periods may also affect resource use, predator avoidance as a metamorph, and phenology as an adult.

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Author Contributions

Conceptualisation: M.K.T.; data collection: J.W., A.W., L.T. & M.K.T; data analysis: J.W., J.R. & M.K.T.; manuscript: J.W., J.R., A.W. & M.K.T.

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