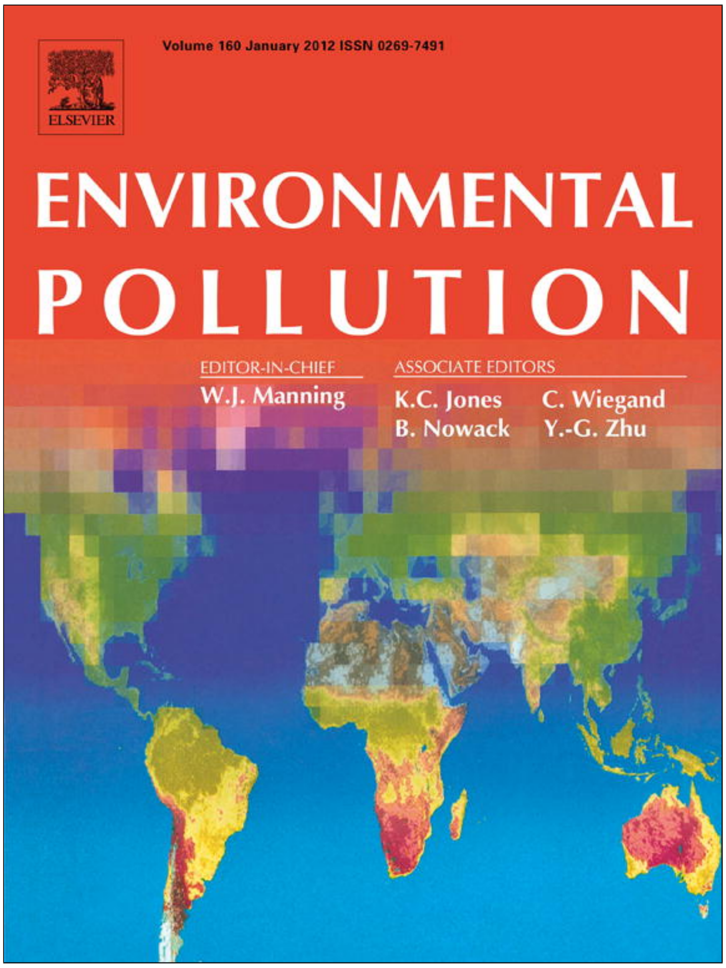
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Increased frequency and severity of developmental deformities in rough-skinned newt (*Taricha granulosa*) embryos exposed to road deicing salts (NaCl & $MgCl_2$)

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A B S T R A C T

Road-side aquatic ecosystems in North America are annually polluted with millions of tons of road deicing salts, which threaten the survival of amphibians which live and breed in these habitats. While much is known of the effects of NaCl, little is known of the second most-commonly used deicer, $MgCl_2$, which is now used exclusively in parts of the continent. Here we report that environmentally relevant concentrations of both NaCl and $MgCl_2$ cause increased incidence of developmental deformities in rough-skinned newt hatchlings that developed embryonically in these salts. In addition, we provide some of the first quantification of severity of different deformities, and reveal that increased salt concentrations increase both deformity frequency and severity. Our work contributes to the growing body of literature that suggests salamanders and newts are particularly vulnerable to salt, and that the emerging pollutant, $MgCl_2$ is comparable in its effects to the more traditionally-used NaCl.

1. Introduction

Approximately 20% of the total land area of the contiguous United States is located within 127 m of a road, and only 3% is located over 5 km away (Riitters and Wickham, 2003). This coverage area of roads has profound ecological consequences (Forman, 2000; Trombulak and Frissell, 2000). For example, amphibian species richness is negatively correlated with road proximity (Collins and Russell, 2009; Houlahan and Findlay, 2003), while simultaneously, probability of skeletal deformities in these animals is positively correlated with road proximity (Reeves et al., 2008). One of the primary ways in which roads affect ecosystems is through the alteration of the chemical environment due to chemical run-off (Forman et al., 2003; Trombulak and Frissell, 2000).

Approximately 14 million tons of road deicing salts are applied annually to roads in North America (Environment Canada, 2001), principally as NaCl and $MgCl_2$ (National Transportation Research Board, 2007; Forman et al., 2003), and salt run-off from these roads has arguably led to the salinization of fresh-water resources in parts of the continent over the last several decades (Kaushal et al., 2005). Roadside habitats have consistently higher Cl^- concentrations than non-roadside habitats (Brady, 2012; Collins and Russell, 2009; Karraker et al., 2008; Turtle, 2000), sometimes reaching limits of up to 4000 mg/L Cl^-, far in excess of the recommended maximum 220 mg/L considered harmful to 10% of aquatic life (Environment Canada, 2001).

A variety of vertebrate and invertebrate organisms inhabiting roadside aquatic habitats are adversely affected by deicing salt contamination (Benbow and Merritt, 2004; Blasius and Merritt, 2002; Collins and Russell, 2009; Petranka and Doyle, 2010). Amphibians, with their permeable skin and eggs are a particularly vulnerable group of animals that live and breed in these habitats. Sodium chloride in particular has been shown to negatively affect survival of all life-stages of frogs and toads (Alexander et al., 2012; Collins and Russell, 2009; Dougherty and Smith, 2006; Harless et al., 2011; Karraker and Ruthig, 2009; Langhans et al., 2009; Padhye and Ghate, 1992; Petranka and Doyle, 2010; Sanzo and Hecnar, 2006; Viertel, 1999). Much less is known of the effects of salt on salamanders and newts. To our knowledge, only one species, the spotted salamander (*Ambystoma maculatum*), has been studied (Brady, 2012; Karraker and Gibbs, 2011; Karraker et al., 2008; Karraker and Ruthig, 2009; Turtle, 2000). Results from these studies suggest that caudates may actually be more susceptible to salt than anurans, but data on other caudate species is needed in order to determine if this pattern holds true.

Compared to work on larval amphibians, relatively few studies have examined the possible effects of road deicing salts on amphibian eggs, despite some evidence suggesting that embryos

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may be more sensitive to salt than larvae (Beebee, 1985; Karraker and Ruthig, 2009; Padhye and Ghate, 1992). In addition to salt killing amphibian eggs, it can also cause embryos to hatch earlier, smaller, and less developed (Gosner and Black, 1957; Hopkins et al., in press; Padhye and Ghate, 1992; Ruibal, 1959), which in itself can have important fitness consequences (Boone et al., 2002; Warkentin, 1995, 1999). Increased developmental deformities, including cysts and spinal and gill malformations, have also been linked to embryonic development in saline water (Gosner and Black, 1957; Haramura, 2007; Harless et al., 2011; Karraker and Ruthig, 2009; Padhye and Ghate, 1992; Ruibal, 1959). Most of these studies, however, only mention deformities anecdotally, and have not quantified frequency or severity. In addition, with the exception of Karraker and Ruthig (2009), who examined *A. maculatum* hatchlings, all studies have focused exclusively on anuran amphibians. Finally, all studies on salt-induced developmental deformities have so far dealt only with NaCl, while the second most commonly used road deicer, $MgCl_2$ (National Transportation Research Board, 2007), has been ignored. While little is known of the effects of $MgCl_2$ on amphibians in general, there is some evidence that it may be at least as, if not more, harmful to anuran tadpoles than NaCl (Dougherty and Smith, 2006; Harless et al., 2011). It is unknown if $MgCl_2$ can cause developmental deformities in amphibian embryos, or how these deformities compare to those seen from NaCl. Given the widespread use of $MgCl_2$ on the landscape (National Transportation Research Board, 2007; Cunningham et al., 2008), and the fact that some agencies are now using it exclusively (e.g., Oregon Department of Transportation, 2012), this represents a significant gap in our knowledge of effects of this contaminant.

The objective of this study was to determine if exposing eggs of the rough-skinned newt (*Taricha granulosa*), a common salamandrid amphibian inhabiting the west-coast of North America, to environmentally relevant concentrations of NaCl and $MgCl_2$ road deicing salts caused developmental deformities in hatchling newts, and to quantify the frequency and severity of these deformities as they relate to salt concentration.

2. Materials & methods

Methods used to rear eggs are based on those presented in detail in Hopkins et al. (2012). This paper should be consulted for detailed methodological explanations regarding rearing newt eggs in petri dishes in the laboratory. Methods regarding the effects of road deicing salts are based on Hopkins et al. (in press), which should also be consulted for more detailed methodologies. Briefly, 16 gravid female newts were collected from the Soap Creek ponds in Benton County, Oregon, in the spring of 2011, and transported back to the lab at Utah State University, where they were induced to lay eggs. These ponds represent a homogenous environment which is not subjected to road deicing salts, as they are located 313 m away from the nearest paved road, which, like all other county roads in this area, is not salted (Kendal Weeks, Oregon Department of Transportation Road Maintenance, personal communication; Kent Mahler, Benton County Road Maintenance, personal communication). In addition, salts are not applied to the nearest highway (over 4 km away) (Kendal Weeks, Oregon Department of Transportation Road Maintenance, personal communication), which is at a similar elevation, and separated from the ponds with a series of hills not conducive to road run-off flow. For more information about this habitat see Gall et al. (2011) and Hopkins et al. (in press).

2.1. Solution preparation and experimental procedure

Eggs from female newts were randomized to 7 different treatments: Low (1.0 g/L Cl^-), Medium (1.5 g/L Cl^-) and High (2.0 g/L Cl^-) concentrations of NaCl and $MgCl_2$ salts and a control (20% Holtfreter's solution; 0.7 g/L Cl^-; a solution recommended for the successful development of salamander embryos (Armstrong et al., 1989)). These salt concentrations were chosen as they are well-within Cl^- concentrations (up to 4.0 g/L Cl^-) measured in road-side ponds impacted by deicing salts in North America (Environment Canada, 2001). Salt treatments were made by mixing pure biological crystalline laboratory grade salts (Thermo Fisher Scientific (New Jersey) – NaCl and Acros Organics (New Jersey) – $MgCl_2$) with distilled water. HCl or NaOH buffer was added in small amounts if necessary to ensure the pH of all solutions was approximately neutral (7.0–7.5). Four milliliters of each salt solution was pipetted into a 3.5 cm diameter, 1 cm deep, round, plastic petri dish, into which 3 randomly assigned eggs from a randomly assigned female were placed. These eggs were then raised at 7 °C in a randomized spot in an environmental control chamber with a 12 h light:12 h dark cycle until hatching. Eggs were checked every day for mortality and hatching, and dead eggs and hatchlings were removed. In addition, we ensured the water level and salinity of the solutions in the petri dishes stayed constant throughout the experiment by drawing a line on outside of the petri dish to indicate the water level, and refilling it with distilled water when necessary to replace water that was lost through evaporation. Adding distilled water ensured that the salinity remained constant, as distilled water has a salinity of 0.0 g/L (G.R.H. unpublished data), and although water may have evaporated from the dishes, the salt would have remained. Water levels were checked and corrected (if necessary) on a daily basis for the duration of the experiment.

2.2. Examination and quantification of deformities

Immediately upon hatching, we transferred each larva to a petri dish with distilled water, and examined it for developmental deformities under an Olympus stereo microscope. We noted the presence of any deformity and characterized its nature. We constructed a list of all deformities observed (Table 1), and characterized each in reference to this list. Each deformity was then scored on an increasing scale of severity from 0 to 3 (0 being the absence of deformity, 3 being a deformity severely detrimental to survival), with animals experiencing multiple deformities being classified according to the most severe deformity present (Table 1). Deformity severity was scored in such a manner based on the deformity's perceived relevance to larval survival.

2.3. Statistical analysis

For our first analysis (presence of deformities), eggs within petri dishes were treated as subsamples, and the petri dish was the replicating factor. The 16 female newts laid 117–594 eggs each (mean ± SE = 300.13 ± 36.02), for a total of 4701 eggs, and these were equally distributed among the 6 different salt treatments. More eggs were placed in control vs. the other treatments, as larvae were needed for a separate experiment on this life-history stage (not discussed here). This distribution of eggs resulted in a total of 859 dishes (3 eggs each) of control, 121 of Low NaCl, 123 Low $MgCl_2$, 122 Medium NaCl, 123 Medium $MgCl_2$, 115 High NaCl, and 118 High $MgCl_2$. A total of 881 eggs died in this experiment (268 in control, 64 in Low NaCl, 49 in Low $MgCl_2$, 89 in Medium NaCl, 90 in Medium $MgCl_2$, 155 in High NaCl, and 166 in High $MgCl_2$). An analysis of the effect of salt treatment on egg survival is provided in Hopkins et al. (in press). For the purposes of the present paper, we analyzed the effect of salt treatment on presence and severity of deformities found on those eggs (3819) that survived to hatching. Eggs from different females were kept separately, and the effect of individual female was treated as a random factor in the analysis. The effect of salt treatment was analyzed as a fixed-effect factor. We used these factors to predict the presence of developmental deformities using a generalized linear model with a binomial distribution and a logit link function with Proc GLIMMIX in SAS®.

Table 1
List of larval deformities and their associated severity score (1 = least severe, 3 = most severe). For animals that experienced multiple deformities, the highest score is used.

Deformity	Score
Slightly bent	1
Small cyst	1
Reduced limb-bud on one side	1
Split tail	1
Bent tail	1
Bent body	2
Cyst	2
Reduced gills	2
Reduced limb-buds	2
Reduced gills	2
Missing limb-buds	2
Very bent tail	2
Very bent body	3
Multiple cysts	3
Large cyst	3
Missing gills	3
Shrunken head	3
Missing eyes	3
Torn/broken yolk sac	3
Missing tail/back end of abdomen	3
Bleeding/bruised	3
Two heads	3
Acephalous	3

Where overall significant effects of salt treatment were found, we conducted Tukey-adjusted pairwise comparisons between treatment levels to compare their influences on deformity presence.

We analyzed the effect of salt treatment on deformity severity score (4 levels: 0, 1, 2, 3) using an ordinal multinomial mixed model with a cumulative logit link; data analysis was generated using the GLIMMIX procedure in SAS®. Salt treatment was incorporated as a fixed effect; female and female by treatment interaction were random effects. It was not feasible to accommodate the clustering of eggs within cups in the model design structure because the high mortality of eggs in salt treatments resulted in a pronounced sparseness of hatchling deformity severity data (see Fig. 1), hence hatchling counts were combined over all cups within each female-treatment combination. Where an overall significant effect of salt treatment was found, we estimated odds ratios for all pairwise combinations of treatment levels (salt concentrations), using a Shaffer-simulated TCTP adjusted *P*-value to judge statistical significance (Westfall and Tobias, 2007; Royen, 1989). Each odds ratio was estimated as the odds of a higher deformity severity score (rather than a lower deformity severity score) for one treatment level relative to the odds of a higher deformity severity score for the other treatment level. An odds ratio of one indicates no difference between treatments.

All statistical analyses were conducted using SAS® software version 9.3 (SAS Institute Inc., Cary, NC, USA), with significance set at $\alpha = 0.05$.

3. Results

A total of 881 eggs died in this experiment, with increasing road deicing salt concentration causing increasing egg mortality (Fig. 1; see Hopkins et al., in press for complete analysis of egg mortality). For those eggs that survived to hatching, there was a significant effect of road deicing salt treatment ($F_{6,1455} = 137.31$, $P < 0.0001$) on the presence of developmental deformities in hatchlings (Fig. 1). Only 6.12 ± 0.67% of newt embryos that successfully hatched in control had developmental deformities, which was significantly less than newt hatchlings in any of the salt treatments (Tukey-adjusted pair-wise comparisons) (Fig. 1). In contrast, 33.58 ± 3.27% and 28.98 ± 3.03% of embryos that survived in low NaCl and low $MgCl_2$, respectively, hatched with developmental deformities (Fig. 1). There was no significant difference between these two salts (Tukey-adjusted pair-wise comparisons), but both were significantly lower in their deformity frequency than eggs that survived in medium salts (NaCl = 65.18 ± 3.40%, $MgCl_2$ = 46.53 ± 3.59%). The majority (73.89 ± 3.59% in NaCl, 60.47 ± 4.08% in $MgCl_2$) of eggs that survived in high road deicing salt concentrations had developmental deformities, with no significant differences in effects between salt types (Tukey-adjusted multiple comparisons) (Fig. 1). Only at medium concentrations were the effects of NaCl and $MgCl_2$ significantly different from each other.

A wide variety of developmental deformities was observed, varying in their severity and frequency (Fig. 2; Table 1). Most deformities were abdominal or spinal, but we also observed many

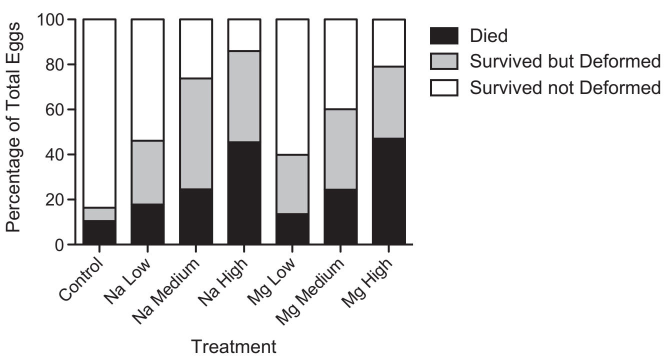

Fig. 1. Fate of *Taricha granulosa* eggs raised in three increasing concentrations of NaCl and $MgCl_2$ road deicing salts, and a control. Black bars indicate the percentage of eggs that died, while gray/white bars indicate eggs that survived to hatching. For those eggs that survived to hatching, increasing salt concentrations caused an increase in the percentage of embryos that hatched with developmental deformities (gray bars), vs. developed normally (white bars) (see Results for relevant statistics).

cases of the reduction or absence of growth of certain tissues (e.g., gills, eyes, limb-buds, and even the head; Table 1). Fig. 2 shows some photographic examples of deformity types.

There was a significant effect of salt treatment on deformity severity score ($F_{6,89} = 66.97$, $P < 0.0001$), with increasing concentrations of both salts generally causing more severe deformities (Fig. 3, Table 2). For example, eggs raised in high NaCl were over 49 times more likely to develop a more severe deformity than eggs raised in control, whereas eggs raised in low NaCl were only 8.79 times more likely to develop more severe deformities than those raised in control (Table 2). There was little difference in the effects on deformity score between the two types of deicing salts, with only medium NaCl having a statistically greater effect on deformity score than medium $MgCl_2$ (Table 2). Nearly 37% of eggs that survived to hatching in high NaCl, and nearly 25% of those hatching in high $MgCl_2$, developed deformities of score 3 (most severe), whereas less than 2% of eggs hatching in control developed deformities this severe (Fig. 3).

4. Discussion

Salt concentrations caused a variety of deformities in newt hatchlings, including spinal deformities (bent tails and bodies), cysts, and shrunken or missing limbs and organs (gills, limb-buds, heads, eyes). In addition, many of these deformities were present in combination with others, presumably adding additional stress to the animal. Other studies examining the effects of salt on amphibian development have noted similar deformities occurring with NaCl. Spinal deformities including kinked tails and curved bodies have been noted in hatchlings of a wide variety of anurans in response to salt (Beebee, 1985; Chinathamby et al., 2006; Gosner and Black, 1957; Padhye and Ghate, 1992; Ruibal, 1959; Sanzo and Hecnar, 2006), as have abdominal cyts (edemas, "swollen bellies"). Shrunken heads (microcephaly) (Padhye and Ghate, 1992) and malformed gills (Ruibal, 1959) have also been noted in anuran embryos exposed to salt. Cysts and spinal deformities in response to salt have been described in the only caudate species studied to date, *Ambystoma maculatum* (Karraker and Ruthig, 2009). Thus, the deformities we describe in newt hatchlings appear to be diagnostic of the effects of salt.

Amphibian egg membranes are highly permeable and sensitive to osmotic changes in salt solutions (Hunter and De Luque, 1959). Road salt (NaCl) approximately equal to our "low" concentrations (~1 g/L Cl^-) has been shown to irreversibly disrupt the osmoregulatory ability of salamander eggs (Karraker and Gibbs, 2011). This disruption caused shrinkage of the perivitelline space (Karraker and Gibbs, 2011), substantially decreasing the area in which the embryo can develop, and has been the suggested cause of curved body and tail deformities seen in anuran hatchlings (Padhye and Ghate, 1992). Physiological stress (increased glucocorticoids) has also been known to result in spinal deformities in fish (Eriksen et al., 2008) and could be partly responsible for the bent bodies and tails we observed in embryos developing in the physiologically stressful salt environments. It makes sense that other deformities we observed, such as large swellings and shrinkages of parts of the developing embryos' bodies are also due to osmoregulatory disruption of the developing embryo, through egg membrane degradation (Gosner and Black, 1957), and/or the inability of developing embryos to osmoregulate (Chinathamby et al., 2006).

We believe that ours is one of the first studies to take a quantitative approach in describing the frequency and severity of different developmental deformities in response to salt, as opposed to simply mentioning their occurrence. Attempting to quantify severity is important because not all deformities are equal in their

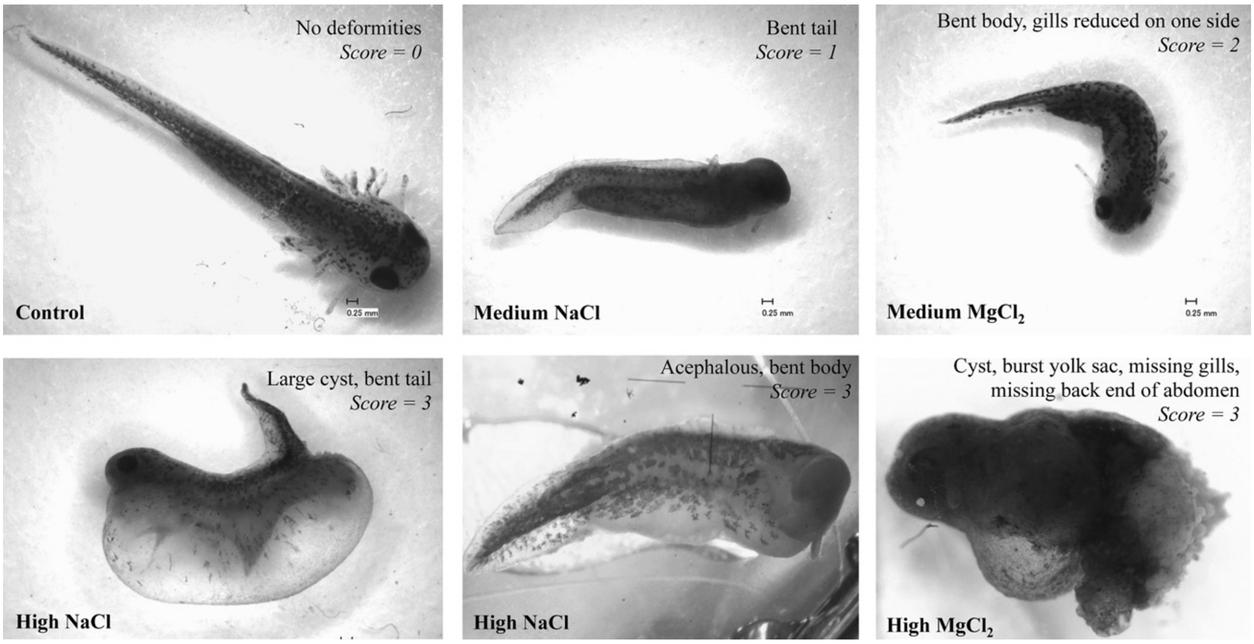

Fig. 2. Photographic examples of developmental deformities in each score class.

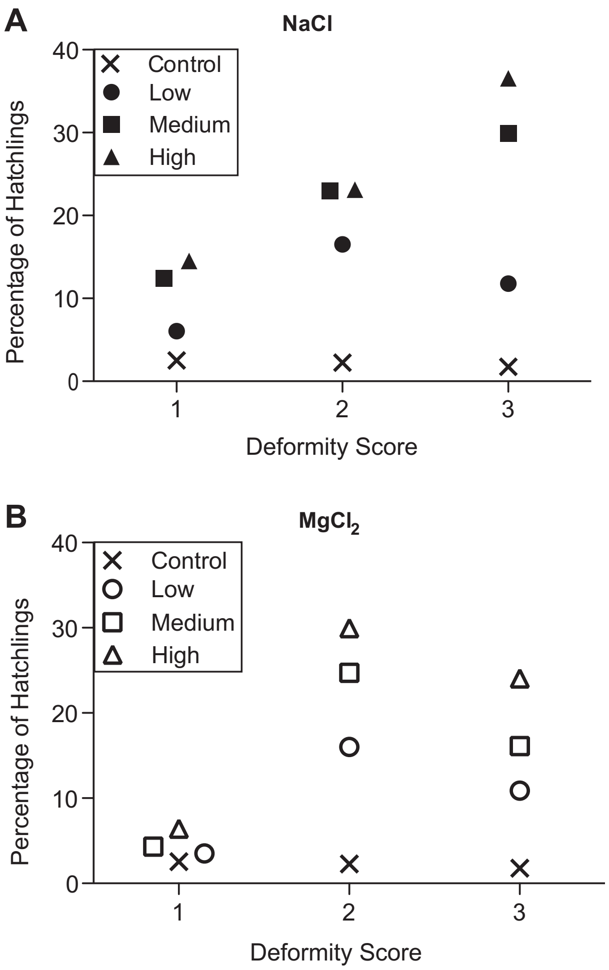

Fig. 3. Percentage of *T. granulosa* hatchlings (those eggs that survived) in each deformity severity score (higher scores indicate more severe deformities), raised in (A.) NaCl, and (B.) $MgCl_2$.

effects on fitness. For example, acephalous hatchlings died within minutes of hatching, while hatchlings with only a small kink in their tail could survive indefinitely. Karraker (2007) found that most frog hatchlings with large cysts (scored as a severe deformity in our study) died within a week of hatching, and the occurrence of this deformity was strongly associated with salt concentration. Eggs developing in higher salt concentrations were significantly more likely to develop severe deformities, with the majority of the most severely deformed individuals in our study developing in medium and high salt concentrations. This gives us important information, often missing in other, more anecdotal studies, regarding the effects of these concentrations on amphibian fitness.

The contamination of amphibian breeding habitats with road deicing salts presents these animals with serious survival problems. Road salts cause eggs to die, and those that survive, to often hatch out sooner, less well developed, at a smaller size, and more deformed (Gosner and Black, 1957; Hopkins et al., in press; Karraker, 2007; Karraker et al., 2008; Karraker and Ruthig, 2009; Padhye and Ghate, 1992; Ruibal, 1959). Nearly all studies on the

Table 2
Odds ratios for pairs of treatment levels based on the cumulative logit model, and tests of whether odds ratios are different than one with *P*-values adjusted for multiplicity using the TCTP method. Each odds ratio is estimated as the odds of a higher deformity score (rather than a lower deformity score) for the horizontal treatment listed in each pair relative to the odds of a higher deformity score for the vertically listed treatment. For example, the odds of a higher deformity score for Na low are 8.79 times greater than the odds of a higher deformity score for Control. An odds ratio of one indicates no difference between treatments.

	Na low	Na medium	Na high	Mg low	Mg medium	Mg high
Control	8.79***	32.61***	49.15***	7.34***	15.36***	28.05***
Na low		3.71***	5.59***	1.20 n.s.	0.57 n.s.	0.31***
Na medium			1.51 n.s.	4.45***	2.12**	1.16 n.s.
Na high				6.70***	3.20***	1.75 n.s.
Mg low					2.10*	3.83***
Mg medium						1.83*

*** = $P < 0.0001$.
** = $P < 0.01$.
* = $P > 0.01 < 0.05$.
n.s. = $P > 0.05$ (not significant).

effects of road salts on amphibians have focused only on anurans. However, the majority of these studies actually did not detect developmental deformities until salt concentrations were used that were far higher than those used in our study (i.e., 4.0–6.0 g/L vs. 2.0 g/L Cl^-) (e.g., Beebee, 1985; Chinathamby et al., 2006; Ruibal, 1959). This concurs with results found for *A. maculatum* (Karraker and Ruthig, 2009) that suggest caudates are more susceptible to salt than anurans. It may aid future research and management decisions to be aware of this apparent discrepancy in sensitivity among amphibian groups.

Our results also suggest that the emerging deicer, $MgCl_2$ appears to be overall as potent in its effects as the traditionally used deicer, NaCl. $MgCl_2$ has become the principal or sole deicer throughout much of the range of *T. granulosa*, and our research, reported here, and elsewhere (Hopkins et al., in press), indicates that its effects are severe on the embryonic development of this amphibian. This chemical deicer is currently the second most commonly used salt in North America (National Transportation Research Board, 2007), and Mg^{2+} has been found to be the most commonly found salt cation in roadside soils in parts of the continent (Cunningham et al., 2008). As the use of $MgCl_2$ increases, more studies should investigate the potentially severe effects of this environmental pollutant on a variety of organisms and life-history stages, to fully assess its ecological impact.

5. Conclusions

Road deicing salts, at environmentally relevant concentrations (Environment Canada, 2001) caused increased frequency and severity of developmental deformities in *T. granulosa* hatchlings. Eggs reared in control solution fared significantly better than eggs in any salt treatment, and increased salt treatment concentration resulted in increased severity of deformities. Results were similar for NaCl and $MgCl_2$, indicating that $MgCl_2$, an emerging stressor, does indeed cause developmental deformities in this amphibian, and at a similar rate as the more traditionally used NaCl. Quantification of the effects of this emerging pollutant on understudied species and life-history stages is critical to ensure the conservation of amphibian populations and biodiversity, especially given results such as ours and others (Karraker and Ruthig, 2009) suggesting that caudates may be more susceptible to salt than more frequently studied anurans.

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