

Effects of Salinity on Early Life Stages of the Gulf Coast Toad, *Incilius nebulifer* (Anura: Bufonidae)

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Anuran amphibian populations worldwide are in decline due to a variety of factors including habitat destruction, climate change, disease, introduction of non-native species, and environmental contamination. We conducted a laboratory trial with *Incilius nebulifer* (synonym: *Bufo nebulifer*) to determine at what level salinity negatively affects hatching and metamorphosis, and how exposure to salinity during development affects metamorph characteristics that influence adult fitness. Embryos exhibited 95.5–99.5% hatching success at salinities of 0, 2, and 4 parts per thousand (ppt); 74.4% success at 6 ppt; and no hatching at 8 or 10 ppt. Salinity affected hatching success and larval survival, and we found linear trends between higher salinity and lower fractions of hatched embryos and living larvae. The odds of hatching were about the same for 0, 2, and 4 ppt, significantly lower for 6 ppt, and zero for 8 and 10 ppt. The odds of survival to metamorphosis were significantly lower in 6 ppt relative to 0, 2, and 4 ppt combined. Time to metamorphosis, mass, and hind limb length of recent metamorphs showed significant differences among treatment groups, with salinity having large effects on these variables. Development time was longer, mass was lower, and hind limb length was shorter in the 0 and 2 ppt treatments compared to 4 or 6 ppt. We showed that salinity affected the survival of early life stages of *Incilius nebulifer* and characteristics that have been linked to adult fitness. Our study suggests that low levels of salinity may affect the survival and fitness of other anurans.

ANURAN amphibian populations worldwide are in decline due to a variety of factors including habitat destruction, climate change, disease, introduction of non-native species, and environmental contamination (Blaustein and Kiesecker, 2002; Peterson et al., 2002; Stuart et al., 2004). Susceptibility to these factors differs among species and populations (Christman, 1974; Bridges and Semlitsch, 2000; Blaustein and Kiesecker, 2002; Gomez-Mestre and Tejedo, 2003; Karraker and Ruthig, 2009; but see Langhans et al., 2009), and depends in part on environmental conditions such as water chemistry (Blaustein and Kiesecker, 2002). Salinity is one parameter of water chemistry that influences the survival, development, and fitness of amphibians, and thus may shape their diversity, distribution, and abundance (Munsey, 1972; Dunson, 1977; Gomez-Mestre and Tejedo, 2005; Smith et al., 2007; Haramura, 2008; Rios-Lopez, 2008).

Most anurans have small migratory ranges compared to other vertebrates, often dispersing no more than 1000 m from their natal sites (Sinsch, 1990), so selection of breeding site depends on the aquatic habitats available within narrow spatial and temporal windows. (Noland and Ultsch, 1981; Sinsch, 1990). Some species choose oviposition sites partly on the basis of salinity (Haramura, 2008), which is a limiting factor for breeding and development even for apparently saline-tolerant species (Beebee, 1979; Andren and Nilson, 1985; Viertel, 1999; Haramura, 2007). Any given species often requires specific environmental conditions for development and metamorphosis of embryos and larvae (Noland and Ultsch, 1981; Werner et al., 2009). Thus, the distribution of adult anurans depends largely on the location of suitable oviposition sites and on the ability of embryos and larvae to survive, develop, and metamorphose at those sites.

Aquatic habitats in coastal areas can experience highly variable incursions of saline water from tides, land subsidence, receding coastlines, and storms, as well as from land

clearing, river regulation, agriculture, de-icing, and construction of levees and canals (Odum, 1988; Allen et al., 1994; Christy and Dickman, 2002; Pardue et al., 2005; Doyle et al., 2007; Petranka and Doyle, 2010). Salinity tolerance in sensitive embryonic and larval stages is therefore particularly important to the survival and distribution of anurans in coastal areas. Although multifactorial field observations help to determine the causes of declining amphibian populations (Blaustein and Kiesecker, 2002), experiments that manipulate one or more environmental parameters are necessary to demonstrate causation (Kefford et al., 2004). A wide variety of salinity trials are represented in the literature, although the results can be difficult to compare due to different methods and salt sources, developmental stages, species, and salinity units (Table 1). To facilitate the comparison of results from the literature, all units were converted to the concentration-based unit parts per thousand (ppt) for the current discussion. The present study used controlled laboratory experiments and a commercially available seawater substitute to determine effects of salinity on survival of embryos and larvae and on characteristics of recent metamorphs of the Gulf Coast Toad, *Incilius nebulifer* (synonym: *Bufo nebulifer*).

Incilius nebulifer is a freshwater species native to the Gulf Coast, from southern Arkansas to Veracruz, Mexico (Mulcahy and Mendelson, 2000). It breeds in temporary water bodies including roadside and irrigation ditches, and coastal marsh pools (Dundee and Rossman, 1989; Hamerson and Canseco-Márquez, 2004), and is assumed to be somewhat salt-tolerant. The population in the present study is not regularly exposed to salt water and, therefore, provides a relatively powerful test of the ability of this species to tolerate future salinity increases. In a two-phase laboratory experiment, we used salinity treatments representing the range found in the local coastal area to ask at what level salinity negatively affects hatching and

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metamorphosis of this species, and how exposure to salinity during embryonic and larval development affects metamorph characteristics that may influence adult reproductive fitness.

MATERIALS AND METHODS

Experimental animals and culture conditions.—Three amplectant pairs of *I. nebulifer* were collected from a ditch in Audubon Park, an urban golf course and public park in New Orleans, LA, USA, during July 2006. There is no evidence of any salt water incursion in this area; the park did not flood during Hurricane Katrina in 2005.

Each pair was placed in a separate bucket of water from the site and allowed to deposit and fertilize their eggs. The adults were then released. The eggs were not assessed for developmental stage at the time they were assigned to treatments. However, all were laid within the same three-hour time span, and assigned to treatments on the same day they were laid.

Three groups of 20–25 embryos from each clutch of eggs were haphazardly assigned to each of six salinity treatments, giving a total of 54 groups (3 clutches \times 3 blocks \times 6 salinities). The salinity treatments were 0, 2, 4, 6, 8, and 10 parts per thousand (ppt), corresponding to approximately 0, 5.7, 11.4, 17.1, 22.9, and 28.6% seawater (% sw), respectively, where 100% sw is 35 ppt. This salinity range reflects that found in the Gulf coastal marshes and the nearby estuarine system of Lake Pontchartrain (Sikora and Kjerfve, 1985; Doyle et al., 2007). Treatments were prepared using dechlorinated tap water and artificial sea salt (Instant Ocean, Aquarium Systems, Inc., Mentor, OH), which provides the same ionic proportions as seawater (Neiheisel and Young, 1992). Salinity of each treatment was confirmed using a YSI-85 Oxygen Conductivity Salinity and Temperature meter (YSI, Yellow Springs, OH).

During Phase I of the experiment, embryos were held in opaque white plastic containers, with a mean and SD of 21.9 ± 0.61 embryos per container (Table 2). A total of 1183 embryos were tested. Each treatment group was held in approximately 237 mL of water, beneath full-spectrum lights on a 12:12 h light:dark cycle in a temperature-controlled room at $21.6 \pm 0.5^\circ\text{C}$. Containers were checked daily, and the water level was kept constant until all larvae had hatched and were free-swimming on Day 7. Dead embryos and larvae were removed when found, and embryo capsules were removed from containers in which all embryos had hatched. Phase I of the experiment ended on Day 7 when the larvae were at Gosner stage 25 (Gosner, 1960) and the fate of embryos (hatched or died) was recorded.

Larvae used in Phase II were haphazardly selected from those alive on Day 7. Due to 100% mortality in the Phase I treatments of 8 and 10 ppt, Phase II included only 0, 2, 4, and 6 ppt treatments. Groups of four larvae were assigned to the same block and salinity treatment in which they had hatched, giving a total of 144 larvae (4 individuals \times 3 clutches \times 3 blocks \times 4 salinities). Larvae were held individually in approximately 250 mL of water in clear plastic cups under the same light and temperature conditions used for embryos. Water level was kept constant, and complete water changes were made every other day when 25.6 ± 1.9 mg of food was added. The food was a 3:1 ratio (by mass) of Geisler Superior Nutrition Diet Rabbit Food (Sergeant's Pet Care Products, Inc.) and TetraMin Tropical

Flakes Clear Water Formula (Tetra Holding [U.S.], Inc.) ground into a powder.

Individual larvae were checked daily, and the emergence of a forelimb was considered the beginning of metamorphosis, whereupon the time to metamorphosis was recorded in days (hereafter referred to as time) and the individual was removed from the salinity trial. Water depth was reduced to 1 cm of dechlorinated fresh water, and a small wad of untreated brown paper towel was added for the metamorph to sit on. Each metamorph was held under the same full-spectrum light and temperature conditions as in the larval stage until the tail was absorbed. Individuals were then blotted and weighed to the nearest 0.01 mg, and snout-to-vent length (SVL) and hind limb length (HLL) were measured to the nearest 0.1 mm using a caliper.

Statistical analyses.—The hypotheses that salinity affected hatching success in Phase I and larval survival in Phase II were tested using the chi-square test and the chi-square test for trend. In this study, embryo and larval survival are given as proportions, and the appropriate way to express the significance of differences between proportions is to use odds ratios (Sokal and Rohlf, 1995). Odds ratios show the probability of an event happening in one treatment relative to another, and can be compared using the chi-square distribution. For Phase I, we used the Mantel-Haenszel test of homogeneity of odds ratios to ask whether the odds of hatching were the same in each treatment relative to the treatment in which the greatest proportion of embryos successfully hatched. For Phase II, we computed the chi-square distributed single squared difference over the estimated variance and used it to ask whether there was a difference in larval survival among salinity levels.

To test for a correlation between mass and the other three variables (time, SVL, and HLL) in Phase II, we performed a Pearson correlation test followed by a comparison of slopes and intercepts equivalent to an Analysis of Covariance (ANCOVA). Chi-square, correlation, and covariance tests were performed using GraphPad Prism version 5.03 (GraphPad Software, San Diego, CA; www.graphpad.com). The hypothesis that mean time, mass, and HLL varied among salinity treatments was tested using Multivariate Analysis of Variance (MANOVA; R version 2.11.1). We power transformed the time data for normality, and included block and clutch as random factors. Data for 17 larvae and 4 metamorphs that died during Phase II were omitted. Univariate ANOVAs were conducted for significant MANOVA results, and effect sizes were calculated by dividing the Sum of Squares for each factor by the total Sum of Squares (Cohen, 1988).

RESULTS

Embryonic hatching success.—In total, 724 embryos (61.2%) hatched and produced larvae that survived to Day 7 (Table 2). There was a significant effect of salinity on hatching success ($\chi^2 = 955.2$, $P < 0.001$), and a significant linear trend between salinity and the fraction of embryos that hatched ($\chi^2 = 759.3$, $P < 0.001$). Fewer embryos hatched in 6 ppt (74.4%) than in the lower salinities (95.5–99.5%), and none hatched at 8 or 10 ppt. The odds of successfully hatching were highest in 2 ppt, with the odds of hatching 6.2 times better than in 0 ppt, 9.4 times better than in 4 ppt, and 68.5 times better than in 6 ppt. We used 2 ppt as the reference for the test of homogeneity of odds

Table 1. Anuran Salinity Studies of Embryos and Larvae, with a Laboratory Component. Due to variation in the definition of 100% seawater (% sw) in the literature, the conversion to parts per thousand (ppt) is given where possible for those studies using % sw.

Family	Species	Developmental stage	Salinity treatments	Salinity effects	Saline source	Reference
Bufo	<i>Epidalea calamita</i>	embryos and larvae from stage 10 ^s	2 to 12 g/L total dissolved solids and a freshwater control	Salinity caused lower rates of survival, development, and growth. Under brackish conditions, the saline population showed higher fitness than the freshwater population.	br	Gomez-Mestre and Tejado, 2003
Bufo	<i>Epidalea calamita</i>	larvae from stage 25 ^s	4, 85, and 140 mOsm	Survivorship and weight did not differ; larvae took longer to metamorphose in > 4 mOsm.	br	Gomez-Mestre et al., 2004
Bufo	<i>Rhinella marina</i>	fertilized embryos (unknown stage)	5 to 50% sw	Salinity caused lower hatching success and higher larval mortality. Embryos did not hatch or develop normally in > 15% sw.	sw	Ely, 1944
Bufo	<i>Duttaphrynus melanostictus</i> ; <i>Fejervarya limnocharis</i> ; <i>Kaboula pulchra</i> and <i>Microhyla ornata</i> ; and Rhacophoridae	larvae (within 8 h of hatching)	0 to 19% sw (100% sw = 35 g/L = 35 ppt)	Salinity caused decreased size and survival in the two microhylids (<i>K. pulchra</i> and <i>M. ornata</i>), but not in the other three species.	MS	Karraker et al., 2010
Bufo	<i>Rhinella marina</i> ; and <i>Leptodactylus albilabris</i>	larvae from stages 23–25 ^s	0 to 12 ppt (32.4–37.5% sw = 14.8 g/L)	At 8 ppt, no <i>L. albilabris</i> metamorphosed and the larvae showed significant weight loss, but 40% of <i>R. marina</i> metamorphosed (accompanied by abnormalities).	IO	Rios-Lopez, 2008
Dicroglossidae	<i>Fejervarya cancrivora</i>	larvae (unknown stages)	20 to 100% sw (100% sw = 930 mOsm/ L = 32 ppt)	Larvae were found in the field in highly saline ponds, but in the lab they did not metamorphose in > 20% sw.	sw	Gordon and Tucker, 1965
Dicroglossidae	<i>Fejervarya cancrivora</i>	larvae from stages 21–22 ^w , 24–25 ^w , and III–XVIII ^{ik}	0 to 32 ppt (100% sw = 980 mOsm/ L = 32 ppt)	Larvae were able to adapt to seawater when acclimation was carried out in steps. Younger larvae tolerated higher salinities than older larvae.	sw	Uchiyama and Yoshizawa, 1992
Dicroglossidae	<i>Fejervarya cancrivora</i>	larvae from stages X–XIV ^{ik}	0 to 100% sw (100% sw = 560 mM Cl)	At constant temperature, survival was > 50% for all treatments < 100% sw.	sw	Dunson, 1977
Dicroglossidae	<i>Fejervarya cancrivora</i> and <i>Hoplobatrachus tigerinus</i>	adults, larvae, and fertilized embryos (unknown stages)	0 to 100% sw (in the field, 100% sw = 36 ppt; in the lab, 100% sw = 35 ppt)	In the field, adults and larvae tolerated salinities up to 39 ppt. But in the lab, embryo development was 10–50% in ≥ 3.5 ppt, with no or abnormal development above that level.	sw or sdss	Gordon et al., 1961
Dicroglossidae	<i>Fejervarya limnocharis</i>	larvae from stages 26–46 ^s	0 to 13 ppt	Larvae metamorphosed at smaller sizes in ≥ 9 ppt, with no metamorphosis above that level.	CL	Wu and Kam, 2009
Hylidae	<i>Litoria aurea</i>	larvae from stages 24–26 ^s	1 to 25% sw (100% sw = 35 g/L = 35 ppt)	The threshold for larval mortality was between 5.5% and 10% sw, but metamorphosis only occurred in < 5.5% sw.	IO	Christy and Dickman, 2002

Table 1. Continued.

Family	Species	Developmental stage	Salinity treatments	Salinity effects	Saline source	Reference
Hylidae	<i>Litoria ewingii</i>	embryos from stages 22–23 ^a	0.14 to 5.6 g/L (16% sw)	Larvae in the highest salinity had lower survival, slower growth, and greater time to metamorphosis, but no difference in mass than freshwater controls. Larvae in low to moderate salinities had greater mass than freshwater controls.	CL	Chinathamby et al., 2006
Hylidae	<i>Litoria ewingii</i>	larvae (four days after hatching)	0.4 to 15% sw (5.25 g/L)	During exposure to salinity, larvae grew slower and were smaller than freshwater controls. Upon return to freshwater, previously salt-exposed larvae grew faster than controls and ultimately did not differ in size.	CL	Squires et al., 2010
Hylidae, and Ranidae	<i>Pseudacris crucifer</i> and <i>P. kalmi</i> ; <i>Lithobates palustris</i> , <i>L. pipiens</i> , <i>L. sylvaticus</i> and <i>L. virgatipes</i>	embryos (unknown stages)	1.5, 2.5, and 3.5 g/L	Salinity caused abnormalities of morphology, locomotion, and activity rate at all treatment levels in the <i>Pseudacris</i> sp. But the <i>Lithobates</i> sp. were unaffected by the lowest treatment, and showed some normal development in the highest treatment.	Hf	Gosner and Black, 1957
Ranidae	<i>Lithobates pipiens</i>	adults and embryos (unknown stages)	1.75 to 9 ppt (100% sw = 35 ppt)	Salinity > 5 ppt was lethal to developing embryos, 3.8–4.6 ppt was semi-lethal, and < 3.8 ppt allowed embryonic development, although abnormalities occurred in > 2.5 ppt. Individuals added to salinity treatments as embryos exhibited higher mortality and slower growth rates at lower salinities than individuals added as larvae. For both groups, mean mass of larvae increased with salinity up to 4 ppt.	br	Ruibal, 1959
Ranidae	<i>Lithobates sylvaticus</i>	embryos from stages 1–5 ⁸	0.375 to 6.00 g/L and a control	Survivorship of larvae was dependent on concentration of NaCl, with mortality occurring sooner in higher concentrations. Acute and chronic exposure caused reduced activity, lower weight, and more physical abnormalities than controls. Chronic exposure also caused lower survivorship and decreased time to metamorphosis in higher compared to lower salinities.	RR	Petranka and Doyle, 2010
Ranidae	<i>Lithobates sylvaticus</i>	larvae from stage 25 ⁸	1.4, 3.5, and 5.6 g/L	Survivorship of larvae was dependent on concentration of NaCl, with mortality occurring sooner in higher concentrations. Acute and chronic exposure caused reduced activity, lower weight, and more physical abnormalities than controls. Chronic exposure also caused lower survivorship and decreased time to metamorphosis in higher compared to lower salinities.	NaCl cert	Langhans et al., 2009
Ranidae	<i>Lithobates sylvaticus</i>	larvae from stage 25 ⁸	0 to 9.75 g/L	Acute and chronic exposure caused reduced activity, lower weight, and more physical abnormalities than controls. Chronic exposure also caused lower survivorship and decreased time to metamorphosis in higher compared to lower salinities.	SF	Sanzo and Hecnar, 2006
Ranidae	<i>Pelophylax perezi</i>	embryos from stages 10–11 ⁸	0, 0.4, and 2 ppt (100% sw = 33 ppt)	Exposure of embryos to fertilizer was lethal only when also stressed by salinity. Fertilizer alone did not cause lethal effects for embryos naturally adapted to saline environments.	NaCl comm	Ortiz-Santaliestra et al., 2010

Table 1. Continued.

Family	Species	Developmental stage	Salinity treatments	Salinity effects	Saline source	Reference
Ranidae	<i>Rana temporaria</i>	embryos from stages 8–9* in intact egg capsules	0.2 to 6.2 ppt	Later stages were more sensitive to sodium than earlier stages. All stages tolerated higher concentrations in the lab than adults selected for spawning in the field.	unk	Viertel, 1999
Rhacophoridae	<i>Buergeria japonica</i>	embryos within 8 h of egg deposition	0 to 5 ppt (100% sw = 33 ppt)	Salinity > 1 ppt caused lower survival rates of embryos, and no embryos hatched in > 4 ppt.	sw	Haramura, 2007

^a Anstis, 2002
^g Gosner, 1960
^{tk} Taylor and Kollros, 1946
^w Witschi, 1956

Saline source abbreviations: (br) brackish water from natural source; (CL) Coralife Marine Salt, Central Garden and Pet, Franklin, WI; (HF) standard Holtfreter solution; (IO) Instant Ocean Synthetic Sea Salt, Aquarium Systems Inc., Mentor, OH; (MS) Meersaltz, Aquacraft Products, Hayward, CA; (NaCl cert) NaCl, Certified for Biological Work, Fisher Scientific, Pittsburgh, PA; (NaCl comm) commercial sodium chloride; (NaCl reag) reagent grade sodium chloride, J. T. Baker Co., Capitol Scientific, Inc., Austin, TX; (RR) Road Runner Ice Melt, Scotwood Industries, Inc., Overland Park, KS; (sdss) sun-dried seasalt; (SF) Sifto non-iodized coarse pickling salt, Canada Inc., Mississauga, Ontario; (sw) seawater from natural source; (unk) unknown.

Table 2. Phase I Mean (± SD) Embryos per Container and Total Number of Embryos Hatched, with Number Died in Parentheses.

Salinity (ppt)	Embryos per container	Total embryos
0	22.1 + 1.54	193 (6)
2	21.3 + 1.22	191 (1)
4	22.0 + 1.73	189 (9)
6	22.6 + 2.30	151 (52)
8	21.8 + 1.30	0 (196)
10	21.7 + 1.50	0 (195)

ratios. As expected, we found that the odds ratios relative to survival in 2 ppt were different among the treatments ($\chi^2_H = 4956120.12$, $P < 0.001$), and therefore we tested separate estimates. The odds were clearly the same in 8 and 10 ppt ($\chi^2_H = 0.65$, $P < 0.001$), and were the same in 0 and 4 ppt ($\chi^2_H = 0.42$, $P < 0.001$), but were different between 4 and 6 ppt ($\chi^2_H = 43.42$, $P < 0.001$) and 6 and 8 ppt ($\chi^2_H = 1562126.05$, $P < 0.001$). The odds of hatching were not significantly different in 0, 2, and 4 ppt, but were significantly lower in 6 ppt, and zero in 8 and 10 ppt.

Larval mortality.—127 (88.2%) larvae out of the 144 selected for Phase II survived to metamorphosis. Of the 17 that died, all but one were from the 6 ppt treatment. There was a significant effect of salinity on larval survival ($\chi^2 = 49.29$, $P < 0.001$), and a significant linear trend between salinity and the fraction of larvae that survived to metamorphosis ($\chi^2 = 29.47$, $P < 0.001$), with fewer larvae surviving in 6 ppt (55.6%) than in lower salinities (99.1%). The odds of survival to metamorphosis were 85.6 times better in the lower salinities combined than in 6 ppt. An odds ratio of 1.0 indicates no difference in outcomes between two treatments (Sokal and Rohlf, 1995). We found a significant difference ($\chi^2 = 14.66$, $P < 0.001$) between the observed odds ratio and an odds ratio of 1.0. The odds of larvae surviving to metamorphosis were significantly lower in 6 ppt relative to 0, 2, and 4 ppt combined.

Time to metamorphosis and metamorph body size.—Four of the 127 individuals that survived to metamorphosis (two each from the 0 and 2 ppt treatments) died of unknown causes and were not included in the analysis of body size. Results suggest that as salinity increased, time increased, mass and HLL decreased, and SVL stayed the same (Fig. 1A–D). Time, SVL, and HLL were significantly correlated with mass ($r^2 = 0.09$, 0.41, and 0.31, respectively, $P < 0.001$). When these variables were regressed against mass, no differences among groups for slope were found ($F = 1.28$, 0.83, and 1.94, respectively, $P > 0.05$), suggesting no interaction effects between salinity and mass. Additionally, no differences among salinity groups for elevation were shown for SVL ($F = 2.27$, $P > 0.05$), supporting the conclusion suggested by Fig. 1C that, independent of mass, SVL was not significantly affected by salinity. However, elevations of the regression lines for time ($F = 31.35$, $P < 0.001$) and HLL ($F = 4.29$, $P < 0.01$) varied among groups and thus these variables were included in the MANOVA.

The MANOVA for time, mass, and HLL showed a significant effect of salinity ($P < 0.001$). Univariate ANOVAs

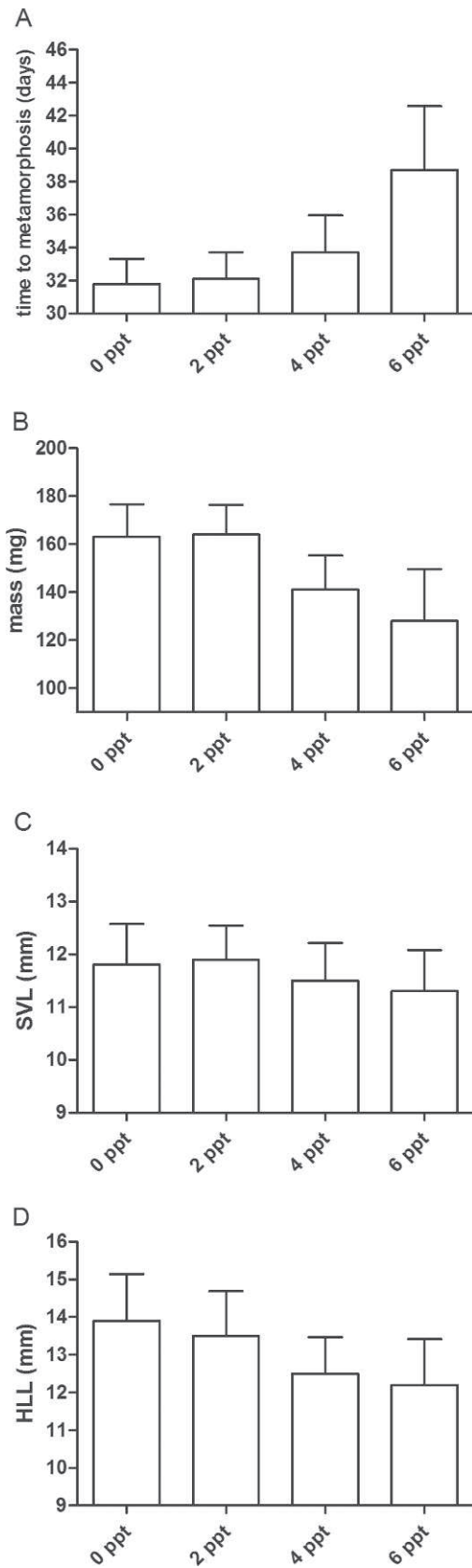


Fig. 1. Phase II mean (\pm SD) results for each of four variables: time to metamorphosis (A), mass (B), SVL (C), and HLL (D). SVL = snout-to-vent length; HLL = hind limb length.

showed that the effect was significant for each of these variables ($P < 0.001$), and calculated effect sizes showed that salinity had large effects on time ($\eta^2 = 0.39$), mass ($\eta^2 = 0.41$), and HLL ($\eta^2 = 0.29$). Tukey's HSD test grouped the 0 and 2 ppt treatments, but kept separate the 4 ppt treatment and the 6 ppt treatment. Time to metamorphosis was significantly longer, mass was significantly lower, and HLL was significantly shorter in the 0 and 2 ppt treatments compared to 4 or 6 ppt.

DISCUSSION

The present study showed deleterious effects of salinity on hatching success and other parameters of *I. nebulifer* above 4 ppt, and a 100% lethal threshold for embryos between 6 and 8 ppt. Compared to some species, *I. nebulifer* exhibited a high mortality threshold for embryos. The mortality thresholds for embryos of *Buergeria japonica*, *Lithobates pipiens*, and *Rhinella marina* have been shown to be 4, 5, and 5.3 ppt, respectively (Ely, 1944; Ruibal, 1959; Haramura, 2007). The range of salinities in which metamorphosis will take place seems wider. Larvae in this study metamorphosed in 6 ppt, although survival was reduced. Comparatively, metamorphosis thresholds for larvae of *Litoria aurea* and *Fejervarya limnocharis* have been shown to be 1.9 and 9 ppt, respectively (Christy and Dickman, 2002; Wu and Kam, 2009).

For larval development, a threshold occurred in the present study between 2 and 4 ppt, when time to metamorphosis increased and mass and hind limb length decreased. Responses to salinity similar to these have been shown in other studies, but the pattern of slower development and lower mass is not entirely consistent in the literature. *Epidalea calamita* has been shown to respond to increased salinity with decreased survival, slower developmental and growth rates, and smaller juveniles entering terrestrial habitats (Gomez-Mestre and Tejedo, 2003). However, this species has also been shown to metamorphose at the same mass in higher salinities (2.9 to 4.8 ppt) compared to very low salinity (0.14 ppt; Gomez-Mestre et al., 2004). *Lithobates sylvaticus* also exhibited the same mass in different treatments, but developed more quickly in response to small increases in salt concentration from 0 to 1 ppt (Sanzo and Hecnar, 2006). Petranksa and Doyle (2010) also found that the mean mass of larvae of *L. sylvaticus* was positively correlated with salt concentration between 0 and 4 ppt. Larvae of *Litoria ewingii* grew more slowly, took longer to metamorphose, and had higher mortality during salinity stress compared to controls (Chinathamby et al., 2006; Squires et al., 2010). But, *L. ewingii* were also found to exhibit higher mass in low to moderate salinities (1.4 to 4.2 ppt) compared to no or high (5.6 ppt) salinity (Chinathamby et al., 2006). Anurans may exhibit compensatory growth after salinity stress as shown by larvae of *L. ewingii* that grew slower during transient exposure to salinities up to approximately 5.3 ppt, then grew faster during the recovery phase, exhibiting no difference in mass upon metamorphosis compared to controls (Squires et al., 2010). The ability to compensate after a temporary period of stress makes sense for species that breed in temporary water bodies and are under selective pressure to use recent growth history as a cue to metamorphic timing (Wilber and Collins, 1973).

Response to salinity stress is stage-specific due to changes in, for example, gill structure, liver function, and hormone

concentrations (Uchiyama and Yoshizawa, 1992; Gomez-Mestre et al., 2004; Wright et al., 2004). Viertel (1999) found that larvae of *Rana temporaria* at Gosner stages 20 to 23 were more sensitive to salinity concentration than earlier stages, though they were exposed for a shorter time period. Larvae of *Fejervarya cancrivora* at the external gill stages (Witschi stages 21–22) were able to survive up to 14 ppt, but advanced larvae with internal gills (Taylor and Kollros stages I–XVIII) were able to survive 17.5 ppt (Uchiyama and Yoshizawa, 1992). Larvae can often survive in higher salinity concentrations than those in which they can metamorphose. In an earlier study of *F. cancrivora*, larvae did not metamorphose in greater than 7 ppt (Gordon and Tucker, 1965). And Christy and Dickman (2002) found that larvae of *Litoria aurea* could survive in up to 3.5 ppt, but required less than 2 ppt to metamorphose.

In the present study, embryos of *I. nebulifer* exhibited deleterious effects of increased salinity at 6 ppt, while larvae and juveniles showed effects at 4 ppt. The only response we measured for embryos was survival, and it is not surprising that lethal effects would be observed at higher salinities than non-lethal effects on development and growth. Somewhat different results might have been found if individuals were first exposed to detrimental salinities at different stages of development. For example, Petranka and Doyle (2010) found higher mortality and slower growth of *Lithobates sylvaticus* at lower salinity levels for individuals introduced to the treatments as embryos than for those introduced as larvae. Furthermore, the eggs used in the present study were oviposited in fresh water. Chinathamby et al. (2006) hypothesized that the lack of immediately apparent negative effects of salinity on late-stage embryos of *Litoria ewingii* in 5 ppt could be due to eggs oviposited in fresh water absorbing a relatively salt-free solution into the egg mass prior to the salinity treatment.

Salinity effects vary among taxonomic groups. Gosner and Black (1957) found that salinity caused abnormalities of morphology, locomotion, and activity rate at all treatment levels (from 1.5 to 3.5 ppt) in two species of Hylidae (*Pseudacris crucifer* and *P. kalmi*), but four species of Ranidae (*Lithobates palustris*, *L. pipiens*, *L. sylvaticus*, and *L. virgatipes*) were unaffected by the lowest concentration, and showed some normal development in the highest concentration. Karraker and Ruthig (2009) also found that embryos of *Lithobates clamitans* were “relatively insensitive” to treatments of road salt compared to embryos of *Ambystoma maculatum*. And while larvae of two microhylids (*Kaloula pulchra* and *Microhyla ornata*) exhibited decreased size and survival above 4.4 ppt, larvae of three non-microhylids (*F. limnocharis*, *Bufo melanostictus*, and *Polypedates megacephalus*) showed no effects of salinity up to 6.6 ppt (Karraker et al., 2010).

Salinity effects sometimes vary even among closely related groups. Adults and larvae of saline tolerant *F. cancrivora* were found to tolerate environmental salinities as high as 28–39 ppt, but the closely related *Hoplobatrachus tigerinus* was found to be a “normal fresh-water frog” in overall salinity tolerance, osmoregulatory responses, and response to desiccation (Gordon et al., 1961). And, Smith et al. (2007) found variation between species within the same family in site occupancy relative to salinity, with a 50% probability of occupancy occurring at 3.5 ppt for *Limnodynastes dumerilii*, while *Limnodynastes peronei* achieved 50% occupancy only at approximately 2.1 ppt.

Incilius nebulifer is likely to encounter salinization in the ephemeral water sources of coastal habitats along the southern United States down into Central America, and farther inland in areas subject to urban and industrial development. The population sampled for this study had likely not been exposed to high concentrations of salinity in the field. Differences in response might be expected in a brackish water population. Gomez-Mestre and Tejedo (2003) found that larvae of *E. calamita* sampled from brackish water ponds had higher salinity tolerance than those sampled from freshwater. However, they also found that all populations shared the same upper limit of 10 ppt.

Although *I. nebulifer* is considered somewhat saline-tolerant (Dundee and Rossman, 1989), results of the present study found deleterious effects of relatively low salinities on survival, development, and growth of embryos, larvae, and juveniles. In nature these effects can impact distribution and abundance. In Australian wetlands, Smith et al. (2007) found that larvae species diversity dropped rapidly above approximately 2 ppt, and was zero above approximately 4 ppt. In northern Puerto Rico, Rios-Lopez (2008) found that abundance of *Leptodactylus albilabris* decreased with increasing salinity along an inland-to-coastal gradient, while abundance of the co-occurring *R. marina* concomitantly increased with increasing salinity.

The present study found that higher salinities resulted in lighter metamorphs with shorter hind limbs. Muscle mass and hind limb length are important characteristics for predicting locomotory speed in anuran amphibians (Choi et al., 2003). Phillips et al. (2006) linked hind limb length to locomotion and dispersal ability in invasive toads (*R. marina*), and concluded that anurans with longer legs have improved dispersal ability. Therefore, an environmental stressor that results in shorter hind limbs for juveniles entering the terrestrial environment may well reduce their ability to disperse.

Salinity effects can also impact individual reproductive success (Leips and Travis, 1994; Bridges and Semlitsch, 2000; Boorse and Denver, 2004). Semlitsch et al. (1988) found that the timing of and size at metamorphosis of juvenile *Ambystoma talpoideum* were directly correlated with size and age of adults at first reproduction. Similarly, *Pseudacris triseriata* that metamorphosed later or at smaller size were less likely to return to the natal pond to breed the first year after metamorphosis (Smith, 1987), reducing their lifetime opportunities for reproduction.

The present study suggests that exposure of early life stages of *I. nebulifer* to salinities above 2 ppt, whether due to natural or anthropogenic events, will increase early mortality, reduce adult fitness, and have an overall negative effect on their distribution and abundance. Even considering the variation inherent in salinity tolerance among taxonomic groups and populations, extending our results suggest that small changes in salinity at critical life stages may affect survival, fitness, distribution, and abundance of many anurans worldwide.

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