Road Salt Impact on a Stream-Dwelling Salamander, Eurycea bislineata: A Low Threshold for Behavioral and Physiological Effects

Kelly Ann Krolik

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Road salt impact on a stream-dwelling salamander, *Eurycea bislineata*: a low threshold for behavioral and physiological effects

By

Kelly Krolik

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Thesis Committee:

Thesis Sponsor Dr. Lisa Hazard

Committee Member Dr. Paul Bologna

Committee Member Dr. Scott Kight
Abstract

Road salt runoff into aquatic habitats is a major ecological issue for amphibian species due to potential toxic effects from chemicals such as hydrocarbons, de-icing agents and salts. Road salt runoff can affect different aspects of amphibian biology, such as osmotic balance, growth, reproduction, behavior, and survival. This study investigated the behavioral and physiological salinity tolerance of adult two-lined salamanders, *Eurycea bislineata*. Two-lined salamanders are found in small streams and rivulets and may be exposed directly to salt runoff. In laboratory behavior trials, *E. bislineata* showed strong aversion to increasing salinity concentrations. They spent less time in higher NaCl concentrations and were more likely to conclude a trial on the control side of the test arena, where conditions were less saline. In physiological performance trials, *E. bislineata* showed reduced stamina (time traveled until exhaustion) and traveled shorter distances as salinity concentrations increased. For both behavioral and physiological trials, negative effects were seen at 0.1 M NaCl and above. Stamina was reduced by 77.5% at this concentration and the EC<sub>50</sub> for behavioral aversion was 0.09 M (95% C.I. 0.012-0.143 M). Salinity levels due to road salt runoff have been found to exceed these concentrations, especially in urban and suburban areas and may pose significant risks for this species. Road salt runoff should be managed to stay under this target concentration to reduce the impacts on amphibians.
ROAD SALT IMPACT ON A STREAM-DWELLING SALAMANDER, EURYcea BISLINEATA: A LOW THRESHOLD FOR BEHAVIORAL AND PHYSIOLOGICAL EFFECTS

A THESIS

Submitted in partial fulfillment of the requirements

For the degree of Masters of Science

by

Kelly Ann Krolik

Montclair State University

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1. Introduction

1.1 Water and Salt Balance

Water and salt uptake is uniquely important for amphibian species. Amphibians have permeable skin, which is a vital feature for the survival of these organisms. One reason why permeable skin is an essential adaptation is because most amphibians do not drink, even when they are dehydrated (Shoemaker and Nagy, 1977, Hillman et al., 2009). Instead, they rely on osmotic influx through the skin for water uptake by changing skin permeability (Shoemaker and Nagy, 1977). Water from streams, ponds, puddles and soil moisture are important sources for the uptake of water by amphibians (Hillman et al., 2009). The rate at which amphibians take up water depends on the species, morphological differences, environmental conditions and other ecological factors (Hillman et al., 2009).

Many amphibian species are found in freshwater habitats, but there are some amphibian species that can tolerate higher salinity environments such as *Fejervarya cancrivora*, *Bufo viridis*, *Xenopus laevis*, and *Batrachoseps relictus* (Hopkins and Brodie, 2015). These species can exist in high salinity environments by accumulating higher concentrations of internal solutes, such as urea, to reduce or eliminate the osmotic gradient that would lead to water loss (Hopkins and Brodie, 2015). In addition, many amphibian species spend time out of the water, which causes loss of water in the organism. In some anuran species, rehydration after loss of body water causes an increase of hydraulic conductivity in the skin (Shoemaker and Nagy, 1977). Other amphibians use a ventral pelvic skin patch that is hyper-vascularized, which aids in rehydration (Hillman et al., 2009).
In addition to water uptake, the skin is also important for salt uptake. Several amphibian species use neurohypophyseal hormones such as aldosterone to control the amount of salt that enters the skin by controlling renal sodium concentrations (Shoemaker and Nagy, 1977). Additionally, it is thought that the storage of electrolytes can help increase sodium levels in the extracellular fluid as a short term acclimation to saline environments (Hillman et al., 2009). Salt uptake through the skin is not the only way amphibians regulate salt concentrations. Larval amphibians start out using their gills as the primary site of salt uptake and once the organism has metamorphosed, the site of primary salt uptake switches to the skin (Shoemaker and Nagy, 1977). Amphibians also gain salt through diet, which varies interspecifically and seasonally (Hillman et al., 2009).

Amphibians must also cope with water loss through evaporation. Evaporation rates vary depending on the environment the organism is found in, as well as the temperature, humidity, and body size (Shoemaker and Nagy, 1977). To reduce water loss, some amphibians that live in a lower humidity environment produce a waxy material composed of lipids that is secreted across their skin to prevent water from leaving their bodies (Toledo and Jared, 1993). Another adaptation to reduce evaporative water loss is the creation of cocoons, as well as the formation of burrows (Toledo and Jared, 1993). In addition, many amphibians, such as anurans, maintain a high body water concentration, but have low extracellular fluid sodium concentrations, which aids in the tolerance to water loss (Hillman et al., 2009, Shoemaker and Nagy, 1977). This adaptation may be the reason why many amphibian species are tolerant to evaporative...
water loss and why terrestrial amphibians can tolerate their water concentrations fluctuating within a wide range (Shoemaker and Nagy, 1977).

1.2 The Effects of Road Salt Runoff

Road runoff is a major ecological issue for amphibian species because it has toxic effects (Sanzo and Hecnar, 2006). Runoff washes contaminants, hydrocarbons, de-icing agents and salts into nearby aquatic habitats. In some regions, salt is the biggest component making up 98% of road salt materials (Sanzo and Hecnar, 2006). This has many implications for the health and survival of aquatic animals, because the salt washes into vernal pools, streams, ponds and rivers, where freshwater organisms are exposed to it. Corsi et al. (2010) found that approximately 16 million tons of road salt are used in North America each year. Additionally, Demers and Sage (1990) showed that salt concentrations in streams near roads can be 31 times higher than streams in rural areas. With such a large amount being used, the effects of this pollutant need to be identified. The effects on terrestrial species are more easily recognized, like vegetation die-back for example. Road salt can affect the survival of other aquatic species, like fish, plants and aquatic invertebrates (Reh and Seitz, 1990). However, there is recent interest in the effects of road salt on amphibian species, which are susceptible to pollution in their environment.

Road salt runoff can have several negative consequences to amphibian species (Hopkins and Brodie, 2015). Amphibians are vulnerable to these salinity fluctuations because they depend on bodies of freshwater for the development of their early life stages
Amphibian eggs develop in water and have no hard shell, which makes them susceptible to environmental changes (Sanzo and Hecnar, 2006). Egg masses of spotted salamanders and wood frogs were less abundant in vernal pools that were closer to the roads due to a higher salinity from road salt runoff (Karraker et al., 2008). In addition, spotted salamander eggs that are exposed to higher salinity water have a high frequency of malformations when hatched (Karraker and Ruthig, 2009).

While amphibian eggs are developing, they use water to aid in oxygen uptake and to increase the space between eggs (Karraker and Gibbs, 2011). Road salt can cause water loss in eggs that are exposed to it that can interfere with development, which has serious implications for the survival of eggs (Karraker and Gibbs, 2011). Survivorship at high salinities was much lower than compared to freshwater eggs (Hopkins et al., 2013). Eggs exposed to high salinity that survived, hatched much sooner compared to eggs that developed in freshwater and were much smaller and less developed (Hopkins et al., 2013). Additionally, tadpoles exposed to road salt show reduced activity, high mortality rates, decreased weight and had delayed metamorphoses (Sanzo and Hecnar, 2006). These results have serious implications for the recruitment and survival of future amphibian populations. If road salt causes a high mortality rate in numerous amphibian species, it might drive populations to local extinction (Sanzo and Hecnar, 2006).

Road salt runoff into aquatic environments is the greatest in the spring when snow melt is the highest (Collins and Russell, 2009). This has major consequences for the breeding of amphibians, especially species that breed early in the spring such as spotted salamanders and wood frogs (Collins and Russell, 2009). These results have
consequences for salt intolerant species because they will not breed in vernal pools with a high salinity, which might affect their populations in the future (Collins and Russell, 2009). Karraker et al. (2008) found that vernal pools within 50 meters of the road have the greatest effects, which might have consequences on the breeding and reproduction of many amphibian species.

While salinity effects on pond-dwelling amphibians have been investigated, little is known of the impact on streamside salamander species. The two-lined salamander, *Eurycea bislineata*, is an abundant aquatic dwelling species that is commonly found in and near streams, so this species could be influenced by road salt runoff. The objective of this study is to determine whether adult two-lined salamanders show aversion to increased salinity and whether salinity affects physiological performance at ecologically-relevant concentrations. Another objective was to determine a threshold for physiological and behavioral salinity effects. This was accomplished by examining the behavioral response and stamina of *E. bislineata* when they were exposed to six different test salinity treatments (0, 0.1, 0.2, 0.3, 0.4, 0.5 M NaCl). This study provides insight on how this species will respond to fluctuations of salt runoff in its environment.

2. Methods

2.1 Study Species

*E. bislineata* is a widespread species found in and near low order streams within the northeastern United States (Barr and Babbitt, 2002). I collected thirty-seven adult salamanders (*E. bislineata*) from Alonzo F. Bonsal Wildlife Preserve in Montclair, New Jersey (40.8500° N, 74.1878° W) that is surrounded by suburban towns. The salamanders were collected in September 2015 and maintained in captivity for 6 months. Animals
were housed in a constant temperature room at 20 °C in groups of 2-5 in plastic containers (29 x 23 x 10 cm) with a sphagnum moss substrate and filled to a depth of approximately 1 cm with aged or dechlorinated municipal tap water. Water was changed and moss was replaced as needed. Salamanders were fed on a regime a diet of wingless fruit flies and small crickets. Research was conducted under IACUC approval (#2015-022).

2.2 Behavioral Preference Trials

The salinity preference of *E. bislineata* was examined using binary choice trials. A test arena was created using a 15 cm diameter plastic Petri dish bisected by a section of 1 ml disposable pipet that was attached to the dish with clear plastic packing tape. The pipet provided a barrier to prevent mixing test solutions, but was low enough that a salamander could easily cross it. Six NaCl treatments (0, 0.1, 0.2, 0.3, 0.4, 0.5 M NaCl) were made by mixing aged tap water and a 1 M NaCl stock solution. A piece of 15 cm diameter filter paper was cut in half and placed inside the test chamber. One half of the filter paper was soaked in the test solution and the other half was soaked in the control solution, which was aged tap water. Solution placement (left/right) was determined randomly for each trial by flipping a coin. The animal was weighed immediately before and after each trial to the nearest 0.01 g. Each individual was placed inside the chamber on the test solution. During a 10- minute trial, I recorded the total time spent on the control and test sides of the arena, the number of times animals moved between sides, and whether the animal ended the trial on the test or control side. The air temperature of the room was also recorded.
Treatments were assigned using a balanced incomplete block design, such that each individual was tested at three of the six concentrations, with a total of 20 trials per concentration. There was a minimum of 31 days between trials for each individual. There were 17 individuals used for the control and 0.5 M NaCl treatment, 15 for the 0.1 and 0.4 M treatments, 16 for the 0.2 M treatment and 18 for the 0.3 M treatment. Differences in the number of individuals tested in each treatment were due to mortality of some individuals.

JMP Pro 11.0 (SAS Institute) was used for statistical analyses using a mixed model ANOVA. For the analysis, total time spent on the test side of the arena was the dependent variable and NaCl concentration was the main effect variable. Room temperature and body mass were included as covariates. The side of the dish that contained the test concentration was randomized by flipping a coin to avoid potential directional bias; test side was therefore included as a factor in the model to confirm that no bias was present. Individual ID was included as a random effect to account for repeated measures on the same individuals.

Activity levels of different species vary, so to facilitate comparisons with prior studies behavioral aversion was converted to a binomial variable and evaluated using logistic regression. When the test concentration is 0 M, paired with a control of 0 M, active species might spend half of their time on each side, while less active species might spend more time on the side they start on. To control for activity level, the average time individuals spent on the defined “test” side at a test concentration of 0 M was calculated (428 sec) and half of that value was used as a threshold for tolerance. For each trial, an
individual was categorized as showing aversion if it spent less than 214 sec in the test concentration, and as showing tolerance if it spent more than 214 sec in the test concentration. This binomial variable was then evaluated using logistic regression, allowing the calculation of an EC$_{50}$ (effective concentration) determining the NaCl concentration at which half of the animals showed aversion.

In addition to time spent on test vs. control sides of the arena, two other measures of behavior were examined. Effect of concentration on the number of times crossed between the test and control sides was tested via ANOVA, and the effect of concentration on the final location (test vs. control) was tested with logistic regression.

2.3 Stamina Trials

To evaluate physiological effects of salinity, swimming stamina (time until exhaustion) was measured at different salinities. To avoid stressing the animals by forcing exposure to hyperosmotic salinities, only lower salt concentrations (0, 0.05, 0.1M NaCl) were used. Solutions were generated as described above. A test arena was created by placing a 10.8 cm diameter glass dish inside of a 38 cm diameter glass dish. The outer dish was filled 100 ml of test solution, resulting in a circular track with a linear distance of approximately 50 cm. The approximate water depth of the test arena was about 3 cm. Prior to initiating the trials, each salamander was measured for weight, total length and the snout vent length (SVL, the length from the tip of the snout to the base of the cloaca). The temperature of the test solution was also recorded. The animal was then placed in the chamber and prompted to swim around the track at a relatively consistent speed by
tapping it lightly on its tail with the recorder’s finger. The time and number of laps swum (to the nearest quarter lap) until exhaustion were recorded. Exhaustion was defined as the absence of movement when prompted until the animal was unresponsive after being tapped lightly 3 times. Each individual was tested once, at a single concentration. There were 8 individuals used for the control and 0.05 M NaCl treatments and 7 individuals used for the 0.1 M treatment. Animals were weighed immediately before and after each trial. JMP Pro 11.0 (SAS Institute) was used for statistical analysis. Because body size can affect locomotion variables, effects of body size (SVL) on distance, speed, and stamina were removed using residual analysis where relevant. The effects of salinity on physiological performance (stamina or time swam until exhaustion, speed, and total distance traveled) were tested using ANOVAs.

3. Results

3.1 Behavioral Preference Trials

There were no significant effects of model co-variates for temperature, body mass, individual ID, or initial placement side (Table 1). NaCl concentration significantly affected behavior, with salamanders at all salinities spending less time in the test concentration compared to the control (Figure 1). To assess the threshold aversion concentration, the logistical regression analysis established an EC$_{50}$ of 0.09M (0.012-0.143 M, 95% CI) and salamanders were more likely to show aversion at higher concentrations ($P < 0.0001$) (Figure 2).

When the activity of salamanders was assessed by number of movements between a salt concentration and the control, no difference was seen in activity level (Table 2,
Figure 3). However, as salinity increased animals were more likely to end the trial on the control side of the test arena (P<0.0001) (Figure 4).

3.2 Stamina Trials

During the initial phase of analyses, two outlier data points associated with control individuals were identified and were eliminated from the model (N= 21). Distance was not affected by SVL (ANOVA; F_{1,19} = 4.35; P=0.0506; R^2=0.186; Figure 5). Stamina was also not affected by SVL (ANOVA; F_{1,19} = 0.354; P=0.558; R^2= 0.018). Speed was affected by SVL (ANOVA; F_{1,19} = 4.35; P=0.0067; R^2= 0.32; Figure 6). Residuals of the regression of speed on SVL were used for analysis of concentration effects.

Distance travelled was not affected by concentration (ANOVA; F_{1,19} = 0.019; P=0.892; R^2=0.001). Residual speed was also not affected by concentration (ANOVA; F_{1,19} = 0.0486; P=0.828; R^2 = 0.0025). Stamina (time until exhaustion) was affected by concentration (ANOVA; F_{2,18} = 3.85; P=0.0405; R^2= 0.299); stamina was lower at 0.1 M than at 0 M NaCl (Tukey’s t-tests, Figure 7).

4. Discussion

These behavior and performance experiments demonstrated that *E. bislineata* behave and perform differently at different salinities. These results are consistent with the hypothesis that that *E. bislineata* could be affected by road salts in the field. I observed that *E. bislineata* showed aversion to salinity, even at low test concentrations (Figure 1). *E. bislineata* were shown to spend less time in the test solution as the concentration of NaCl increased. This study is especially important because it connects both behavior and physiological performance to increased salinity. It also focuses on a stream dwelling
species, while previous amphibian salinity research has mainly focused on vernal pool ecology.

*E. bislineata* were observed to show aversion to salinity at 0.1 M NaCl, which may have major ecological implications for this species, as well as other amphibian species. Road salt runoff in suburban and urban streams and lakes has been found to reach 0.125 M, which is higher than most amphibians can tolerate (Kaushal et al., 2005). Similar studies showed Eastern Newts, *Notophthalmus viridescens*, have a similar aversion to high salinities and they were found to be sensitive to salinities of 0.2 M NaCl and higher (Kwasek, 2011). It is hypothesized that Eastern Newts are more tolerant to saline habitats to decrease the risk of contracting parasites or because of osmoregulatory preferences (Kwasek, 2011). Spotted salamanders and wood frogs are also behaviorally sensitive to increased salinity (Collins and Russell, 2009; Hazard et al., unpublished). These species are shown to be absent from habitats with high salinity, which can have implications for the reproduction of these organisms (Collins and Russell, 2009). Species that are selective in spawning sites will be greatly affected by increased road salt runoff because it will limit breeding locations (Viertel, 1999). In addition, breeding sites with high salinity have also caused a high mortality rate in larval amphibians, which could affect recruitment and lead to local extinction (Karraker and Ruthig, 2009).

This experiment also demonstrated that high concentrations of salt can impact the performance of *E. bislineata*. Exposure to salt at the highest concentration, 0.1M NaCl, reduced stamina to 77.5% of control levels which also affected the distance the animal traveled. Similar results have been shown in *Rana temporaria* tadpoles, in which higher
salinity caused a decrease in the swimming speed and overall movement (Denoël et al., 2010). In addition, *Rana breviceps* tadpoles were shown to have reduced swimming activity, along with loss of balance with increased salt exposure (Mahajan et al., 1979). Reducing an organism’s stamina could cause it to become more susceptible to predation and reduce its ability to catch prey (Austin and Shaffer, 1992).

Salamanders and other amphibians are mid-level predators so they form an essential part of the food web and have been shown to be a key regulator of invertebrates in aquatic habitats (Davic and Welsh, 2004). In addition, salamanders are a food source for higher level consumers, such as birds, turtles, fish and mammals. Exposure to salt could lead to changes in not only the population of *E. bislineata*, but also other species that prey on and that are consumed by *E. bislineata*. Reductions in stamina are expected to reduce foraging/predation efficiency, which could also influence community dynamics and trophic interactions in the food web. Additionally, limiting the distance the animal can travel can reduce its ability for migration and dispersal and also reduce the animal’s likelihood to escape sudden salt influx from spring thaw (Austin and Shaffer, 1992). *E. bislineata* are known to migrate during breeding season and can disperse up to 100 meters from the streams which they inhabit. Reductions in stamina could influence the dispersal of this species along with the local population ecology because it could affect the reproductive output and the local distribution of the species.
Management strategies

These results suggest that *E. bislineata* shows behavioral and physiological responses to short term salinity exposure at and above 0.1 M NaCl. The results for both time spent in the test concentration and stamina are also consistent with a threshold less than 0.1M NaCl and I was able to establish an EC$_{50}$ of 0.09M (Figure 2). As such, streams that are influenced by road salt runoff should be managed to stay under this target concentration. Potential effects of long term exposure, even with lower salinity concentrations, should also be considered for the management of road salt runoff. Protecting stream habitats near roadsides would help reduce mortality and improve the reproduction and recruitment of amphibians and their offspring. In addition, road salt use should be reduced in times of breeding so that breeding sites are less susceptible to an increase in salinity.

Another strategy to solve this problem might be to engineer a de-icer that will not be so detrimental to amphibians and other organisms. Alternative de-icers such as urea, calcium magnesium acetate and potassium chloride have been suggested because they are just as effective and cost effective; however, the effects on amphibians have not been well researched (Ramakrishna & Viraraghavan, 2005). These de-icers have other known environmental effects, such as the contamination of above and below ground water supplies (Fu et al., 2012). A better alternative might be sugar molasses products, such as beet juice. Beet molasses is less corrosive and less harmful to wildlife (Fu et al., 2012). It can also be mixed with other de-icers, such as road salt (Fu et al., 2012). The best management strategy would take in account the cost efficiency of road de-icers, the
impacts of road de-icers not only on amphibians but other organisms and the reduction of human fatalities and injuries.

**Conclusions**

Road salt runoff is a major ecological problem for amphibian species. It causes increased mortality, slows growth and development, alters community structure, and affects the behavior and physiology of many amphibian species. This experiment also showed that stream dwelling species, such as *E. bislineata*, are sensitive to road de-icers concentrations higher than 0.09 M NaCl; where it affected both their behavior and physiology. To help protect amphibian populations, road salt runoff should be managed to stay under this target concentration to reduce the impacts of road salt on amphibians and other aquatic organisms. In addition, the amount of runoff in streams should also to be monitored in times spring thaw to reduce the exposure of de-icers to stream dwelling species. Road salt runoff has become a major issue for amphibian species and solutions to this problem must be solved before there are irreversible ecological consequences.
Table 1. Results of the ANOVA for behavioral aversion of two lined salamanders to salinity. Total time spent on the test side of the arena was the dependent variable; NaCl concentration was the main effect variable. Ambient temperature, body mass and potential side bias (left or right) was also accounted for. Overall ANOVA: $R^2 = 0.554$, N= 99, Individual ID accounted for 12.5% of the total variance.

<table>
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<th>Source</th>
<th>D.F.</th>
<th>D.F. Error</th>
<th>F Ratio</th>
<th>Prob &gt; F</th>
</tr>
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<tbody>
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<td>NaCl Concentration (M)</td>
<td>5</td>
<td>80.19</td>
<td>5.4959</td>
<td>0.0002</td>
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<td>Temperature (°C)</td>
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<td>80.19</td>
<td>0.1496</td>
<td>0.6999</td>
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<td>Pre-Trial Mass (g)</td>
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<td>0.864</td>
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<tr>
<td>Test Solution on Left or Right</td>
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<td>84.56</td>
<td>0.9854</td>
<td>0.3237</td>
</tr>
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</table>
Table 2. Results of the ANOVA for activity level (number of times crossed between test and control sides) showing the amount of movement by the animals at different concentrations. Overall ANOVA: $R^2=0.148$, N=99, Individual ID accounted for 4.14% of the total variance.

<table>
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<tr>
<td>Test Solution on Left or Right</td>
<td>1</td>
<td>88.45</td>
<td>2.0657</td>
<td>0.1542</td>
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Figure 1. The behavioral aversion of *E. bislineata* to NaCl. Time spent in each treatment (mean ±S.E.) during a 10-minute trial. Columns sharing letters are not significantly different from one another (Tukey’s T-tests *P*<0.05)
Figure 2. Proportion of individuals that showed NaCl tolerance during a 10-minute choice trial to establish the EC$_{50}$ of aversion using logistical regression ($R^2 = 0.312$; $P < 0.0001$). The calculated EC$_{50}$ was 0.09 M with arrows representing the 95% confidence interval.
Figure 3. The effect of NaCl concentration on activity level of *E. bislineata*, measured as the average number of times the animal crossed (mean ±S.E.) between the test and control sides of the arena.
Figure 4. The probability of ending an individual choice trial on the side with the test solution. The green line represents the data points and the blue line represents the trend line. $P < 0.001$, $R^2 = 0.3116$, $N = 99$
Figure 5. The effect of body size (snout-vent length, SVL) on distance traveled by *E. bislineata*. Linear regression, $P = 0.0506$, $R^2 = 0.19$, $N = 21$
Figure 6. The effect of body size (snout-vent length, SVL) on swimming speed of *E. bislineata*. Linear regression, $P=0.0067$, $R^2=0.32$, $N=21$
Figure 7. The stamina (time to exhaustion) of *E. bislineata* swimming in different NaCl concentrations during physiological performance trials (mean ±S.E.). Treatments sharing letters are not significantly different from one another $P = 0.040$, $R^2 = 0.30$, $N = 21$
References


