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Behavioral Aversion of Two Ranid Frogs to Road Deicers : Does Terrestriality Influence Sensitivity?

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Behavioral Aversion of Two Ranid Frogs to Road Deicers: Does Terrestriality Influence Sensitivity?

by

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Abstract

Amphibian decline has been a global phenomenon in recent decades due in part to negative human effects on the environment. Sensitivity of amphibians to environmental degradation makes them good indicators of environmental change, attesting to the importance of amphibian inventory and population monitoring. De-icing salts, such as sodium chloride, are applied to roads during the winter months and may accumulate in aquatic and terrestrial habitats. The exposure of amphibians to high salt concentrations can impair their physiological functioning due to their highly permeable skin. The purpose of this investigation is to examine the behavioral tolerance threshold for road salt (NaCl) for the green frog (*Rana clamitans*) and wood frog (*Rana sylvatica*). These species were chosen based on levels of terrestriality; green frogs spend most of their time in or near water, while wood frogs breed in vernal pools and spend the rest of the year in moist woodlands. In 10-minute trials, the frogs were allowed to choose between a NaCl solution ranging from 0 to 0.5 M concentration and a control solution (aged tap water, 0 M) to examine their behavioral tolerance to saltwater; mass change due to osmotic water flux was also measured. Despite the close relatedness of the two species, evidence shows that their salinity threshold varies. Green frogs showed an aversion to increasing NaCl concentrations. However, the wood frogs showed no aversion during the 10-minute trials and little to no aversion during subsequent 20 and 30-minute trials. Differences between these species may be due to higher tolerance of dehydration in wood frogs due to their more terrestrial nature or more specifically due to their tolerance of increased internal osmolarity as a part of their mechanism for tolerating freezing conditions.

BEHAVIORAL AVERSION OF TWO RANID FROGS TO ROAD DEICERS:

DOES TERRESTRIALITY INFLUENCE SENSITIVITY?

A THESIS

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Introduction

Amphibian decline has become a global phenomenon in recent decades due to negative effects on the environment (Karraker and Ruthig 2009). There are many causes for this gradual decline. Climate change, soil acidification, UV-B exposure, and pathogens account for some of the decline (Collins and Russell 2009). One underlying cause is the human population growth that is increasing throughout the world, leading to development of cities, buildings, and roads. Some of the effects of road and other construction include loss of habitat, isolation of populations, increased edge effects, barrier movements and gene flow (Sanzo and Hecnar 2006). The increase in roads results in more cars, leading to more transportation and the eventual dramatic increase in pollution and runoff.

In winter months, de-icing agents are applied to roadways to decrease the risk of accidents. They work by lowering the freezing point of water and act as a melting agent by preventing ice from bonding to the pavement (www.saltinstitute.org). Sodium chloride (NaCl), calcium chloride ($CaCl_2$), magnesium chloride ($MgCl_2$) and potassium acetate are all salts used for de-icing (Novotny et al. 2008). The most commonly used salt is sodium chloride (NaCl), because it is easy to store and fairly inexpensive. It has been estimated that the annual increase in salt used for deicing roads has increased from 163,000 tons in 1940 to over 23 million tons in 2005 and 35% of all domestic salt demand is used for deicing (Novotny et al. 2008, United States Geological Survey).

Salt, along with metals and hydrocarbons, are all part of the roadway run-off that contaminates the environment (Sanzo and Hecnar 2006). During the winter months between November and April, salt concentrations tend to be higher than normal. Roads are salted on a regular basis during the winter months, and the salt concentrations tend to build up and accumulate as it is spread over the roads. As temperature increase, the salt runoff ends in streams, rivers, ponds, storm drains, and eventually reaches the groundwater, affecting the soil and water chemistry (Novotny et al. 2008). Normal background chloride concentrations are typically only a few milligrams per liter, with some variability resulting from topography, geology, and geographic location (Sanzo and Hecnar 2006). Naturally occurring chloride levels in groundwater are typically less than 10 mg/L, and can range from 0 to 100 mg/L in surface waters (Jones and Jeffrey 1992, Goldman and Horne 1983). Sodium concentrations have a much lower range, and according to the United States Environmental Protection Agency, have to remain less than 20 mg/L in drinking water (U.S. Environmental Protection Agency 1998).

A study from Environmental Canada, Canada's Environmental Protection Agency program, has reported road runoff concentrations that exceed 18,000 mg/L. They have also found that concentrations can range from 150 mg/L in rural lakes to 5,000 mg/L in urban impoundment lakes. Ponds and wetlands can reach concentrations of 4,000 mg/L and watercourses can reach 4,300 mg/L (Environmental Canada 2001).

There have been noticeable changes in amphibian physiology and survival such as nervous system problems, mortality in all life stages, altered behavior, and compromised growth and development because of these issues (Karraker and Ruthig, 2009). Effects from other metals and other types of pollution have been well studied, but consequences of exposure to road salts have not been investigated as much.

Some studies have shown that sodium chloride has had little effect on normal amphibian physiology, while others have shown that it has caused mortality and other harmful effects on both plants and animals. Experiments have shown that a higher salt content has caused physiological problems in various amphibian eggs and tadpoles. For example, Denoal et al. (2010) found that high NaCl concentrations affected tadpole behaviors; tadpoles at high concentrations moved at slower speeds and across shorter distances than at low concentrations. This can be detrimental because it may prevent the frogs from escaping predation. Sanzo and Hecnar (2006) performed a similar experiment with larval wood frogs. They found that tadpoles had decreased survivorship, weight, and activity levels, metamorphosed earlier and showed increased developmental abnormalities. They also found that species richness increased with increased distance from the closest road, which shows that as salinity decreases with increasing distance from roads, survivorship also increases. Decreased activity levels were also observed; reduced activity and feeding, delayed responses, emaciation, and tadpoles had developed bent and disintegrated tails (Sanzo and Hecnar 2006). Researchers found that at the control, low, and medium treatments survivorship was about 37%, but at high treatment levels, survivorship was only 17%. These results show that increasing NaCl concentrations results in negative and fatal effects on wood frogs in the larval and tadpole stages.

In adults, the main initial effect of exposure to high salinity is dehydration due to osmotic loss of body water. Due to potential lack of readily available water, terrestrial frogs have adapted mechanisms to conserve as much water as possible (Churchill and Storey 1993). Terrestrial and semi-aquatic species can tolerate a 25-60% total body water loss without any harm (Hermes-Lima and Storey 1998). I predicted that the more aquatic green frogs would show more aversion to an increased environmental salinity than the more terrestrial wood frogs because they would be less tolerant of dehydration. I also predicted that with increasing salinity and longer time spent in test solution, frogs would lose more mass.

Green frogs and wood frogs were the focus of this study. Green frogs (*Rana clamitans*) are very common in North America, including Canada. They have a wide range from the greater New England area to Georgia in the east, and from southern Canada southward into Oklahoma and Arkansas (Hulse, McCoy and Censky, 2001). They are very active in comparison to many other frogs, especially during the breeding season. Seasonal activity usually ranges from March to October and hibernate the remaining months (Hulse, McCoy and Censky, 2001). These frogs inhabit a wide variety of shallow fresh water areas including springs, creeks, rills, ditches, vernal ponds, small streams, and along the edges of lakes and ponds (Conant, 1975).

Green frogs are most active during reproduction, which begins at the end of April and can continue all the way through August (Hulse, McCoy, and Censky, 2001). Amplexus and breeding occurs in the water, as well as hatching and the beginning stages of metamorphosis. Egg masses can contain between 3,000 and 5,000 eggs and females can lay one or two masses per breeding season depending on weather condition. Depending on weather conditions and time of egg laying, development from hatchling to metamorphosis can range from 70-365 days. Hatching usually occurs within 3-5 days; the tadpoles stay in the area for one to two days then begin to forage the water column. However, if eggs are laid late in the season, tadpoles will overwinter and metamorphose the following summer (Hulse, McCoy, and Censky, 2001).

Wood frogs (*Rana sylvatica*) are quite different from green frogs. They are a more terrestrial species, and only live in water when breeding. Also inhabiting North America and Canada, they can be found as far west as Alaska, east in Labrador, and south into Georgia (Hulse, McCoy, and Censky, 2001). They are mainly found near moist wooded areas in lowland deciduous forests (Conant, 1975; Hulse, McCoy, and Censky, 2001). They have one of the longest activity periods of any northeastern anuran because they emerge from hibernation as early as February and can be active until October (Hulse, McCoy, and Censky, 2001).

Hibernation begins in October and wood frogs can be found in shallow burrows, which are usually above the soil frost line in the winter months. Because of this, wood frogs risk extreme temperatures and freezing to death. To accommodate this, they have developed freeze tolerance, and can withstand temperatures as low as -7°C. This occurs by allowing the liver to produce increased levels of glucose, which lowers the freezing point of the body, and reduces the risk of death (Hulse, McCoy, and Censky, 2001).

Due to an early emergence, reproduction is also started earlier. Reproduction lasts about a week and occurs inside ponds, roadside ditches, canals and flooded burrow pits. Females tend to lay egg masses together to increase changes of development; each clutch can have up to 745 eggs. After 20 days of incubation, tadpoles begin to hatch and stay in the area for 1-2 days until they begin to feed on the bottom, which can last up to 113 days (Hulse, McCoy, and Censky, 2001). Since developmental time varies depending on weather conditions, tadpoles may be exposed to high salt concentrations for about 6-15 weeks before metamorphosis (Harding, 1997).

I tested the hypothesis that green frogs would show a higher aversion to increasing salt concentrations than the wood frogs using behavioral choice trials between a control solution and a range NaCl concentrations.

Materials and Methods

Study Organisms and Maintenance

Wood frogs (*Rana sylvatica*) and green frogs (*Rana clamitans*) were collected from vernal pools at the School of Conservation in Branchville, New Jersey. All frogs used were captured in spring of 2010 or 2009.

Laboratory temperatures ranged from 18.8 to 23.0 °C, and barometric pressure ranged from 746.7 to 755.8 mmHg. Indoor lighting reflected that of natural conditions. All frogs were fed crickets every 2-3 days, and water was changed as needed.

All frogs were kept in glass terrariums with shredded coconut bark bedding. Ultraviolet lights (Powersun 10.0, ZooMed) were used about 10 hours per day to simulate natural conditions.

Behavioral Tolerance

To determine their behavior tolerance to sodium chloride (NaCl), 10 green frogs and 8 wood frogs were given a choice between a solution of NaCl and aged tap water during a 10-minute choice trial. The NaCl solutions tested were 0.05 M, 0.10 M, 0.15 M, 0.20 M, 0.25 M, and 0.50 M. The aged tap (0 M) was aged for a minimum of 24 hours or de-chlorinated. Two 15.24 x 15.24 centimeter trays were placed side by side in a standard 18.93-liter fish tank. There was no excess space left around the trays, so the animal was forced to choose between the two solutions. Dark paper towels covered the exterior of the fish tank so any outside movement or activity would not interfere with the frogs' behavior. A mirror was set up below the tank to observe the frogs' movements without disrupting them.

Test subjects, solutions, and initial placement (whether test solution would be in right or left tray) were randomized. No subject was used twice any sooner than three days from the previous trial. Test trays were filled with 500 ml of either aged tap water or salt solution. Urination prior to weighing was attempted, but did not always occur. Test subjects were blotted dry, weighed, and placed into the test solution. Behavior was then recorded for 10 minutes. Major movements such as moving, changing positions, escape attempts, tray changes, and any other significant movements were noted. Following each timed trial, subjects were dried and re-weighed, and the trays, tank, and scale were washed with aged tap to decrease the chance that any chemical cues would influence the next frog's behavior.

Upon completing all 10-minute trials and analyzing results for any patterns, 20-minute ($N = 5$; three frogs had died since the 10-minute trials) and 30-minute trials ($N = 5$; one frog died and was replaced with a new capture) were tested using the wood frogs only. The frogs were exposed solely to 0.50 M solutions and behaviors were recorded as previously described. Following the 30-minute trials, righting response was also recorded in order to determine if muscle functions were affected by salt exposure. Righting response was measured by turning the frogs on their backs immediately after each trial and the amount of time required for them to turn back over was recorded.

Data Analysis

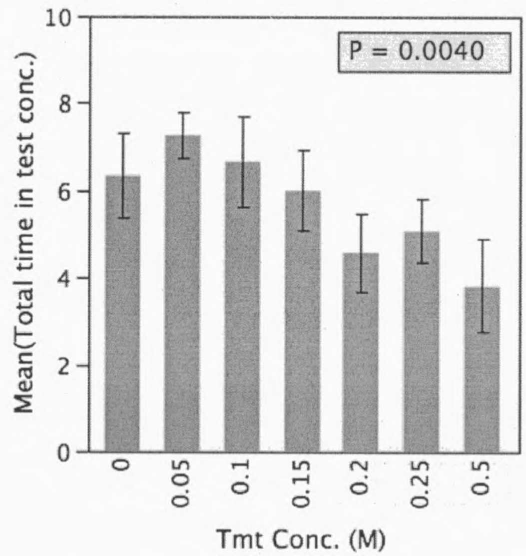
Data were analyzed using JMP 8.0 for Mac (SAS Institute). A P-value of less than 0.05 was considered statistically significant. Mass change was analyzed using linear regression. Movements and escape attempts were analyzed via t-test. Mean time spent in the test concentration is presented for informational purposes. However, data were not normally distributed and therefore time was converted to a binary measure, aversion/tolerance, for analysis using logistic regression.

Aversion was defined as spending less than half of the time spent in the test concentration as was spent in the control concentration (0 M). The aversion threshold time was different for each species. Green frogs stayed in the 0 M control solution for an average of 6.33 minutes, so 3.16 minutes was used as the boundary between aversion and tolerance. The wood frogs stayed at 0 M for an average of 9.03 minutes, so 4.52 minutes was used as the threshold.

Results

10-minute choice trials

With increasing environmental salinities, green frogs spent less time in the test solution before moving to the aged tap water (Figure 1A). The mean time spent in 0.05 M was 7 minutes, while the mean time spent in 0.5 M was 4 minutes. The wood frogs show a lower aversion to higher environmental salinities than the green frogs. Of the eight wood frogs observed, all of them spent a mean time of 8:30 or more in all test solutions ranging from 0 M to 0.5 M (Figure 1B).



A.

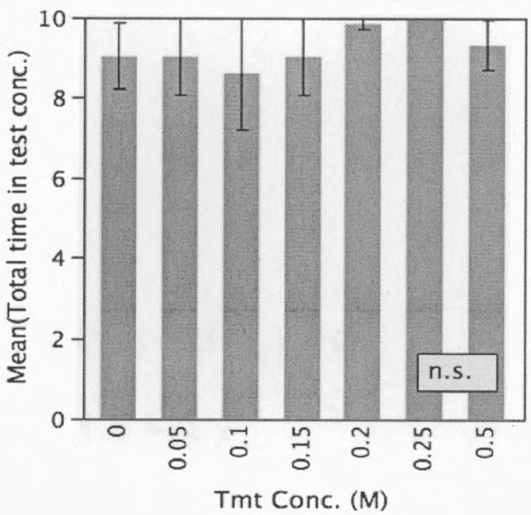


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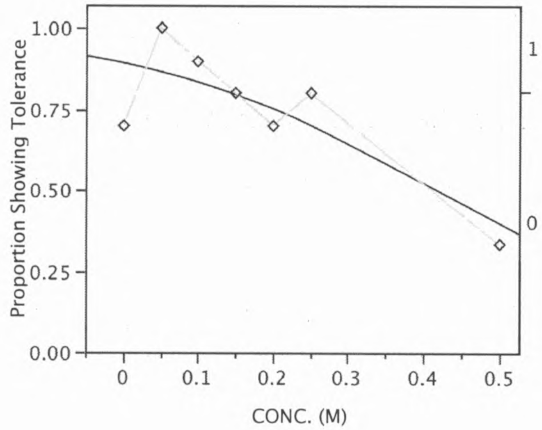
Figure 1: Mean total time spent in the test concentration during 10-minute choice trials. (A) Green frogs. (B) Wood frogs.

For the more active green frogs, we found a significant increase in aversion with increasing concentration (Figure 2A; logistic regression P = 0.004). On the other hand, the wood frogs showed no aversion to salts; more than 75% of the frogs overall stayed in the test solution for over the threshold time for that species, and there was no effect of concentration on probability of aversion (Figure 2B; logistic regression, P = 0.093). EC_{50} is the predicted concentration at which 50% of the animals showed aversion. The green frogs showed an EC_{50} of 0.417 M (95% confidence interval of 0.086 to 0.591). Because there was no significant effect of concentration for wood frogs, we were unable to calculate an EC_{50}.



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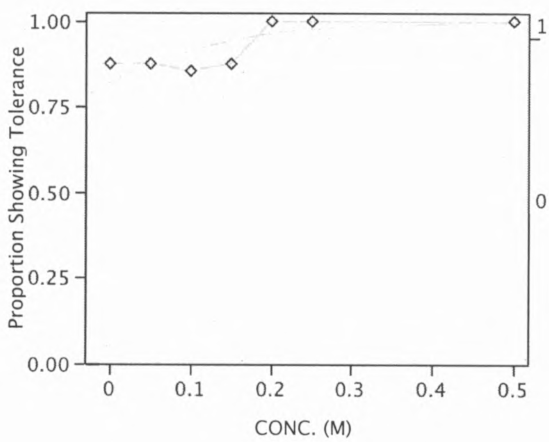

B.

Figure 2: Proportion of frogs showing tolerance during 10-minute choice trials. Raw date (solid) and logistics curve (dashed) calculated. (A) Green frogs show aversion as defined by spending less than half of the time spent in 0 M concentration. (B) Wood frogs show no aversion.

20 and 30-minute choice trials

With no significance seen in the 10-minute trials for wood frogs, they were exposed to longer timed choice trials at the highest concentration, 0.5 M NaCl. Mean time in the test solution for the 20-minute trials was 16 minutes (Figure 3). Three of the five frogs spent the entire 20 minutes in test solution, one spent less than ten minutes, and one spent about 14 minutes in test solution. For 75% of the time, they sat, with little to no movement in 0.5 M NaCl solution. Once again, the wood frogs showed very little aversion to a high environmental salinity. Righting response was immediate; indicating no physiological effects.

Mean time in test solution for the 30-minute trials was 22 minutes (Figure 3); about 70% of the time, they sat, with little or no movement in 0.5 M NaCl solution. Three of the frogs (3/5) spent the entire 30 minutes in test solution. The other two frogs moved from the test solution in less than 12 minutes. Adult wood frogs continued to show little aversion to higher environmental salinities. Once again, righting response was immediate for all frogs, indicating no negative physiological effects.

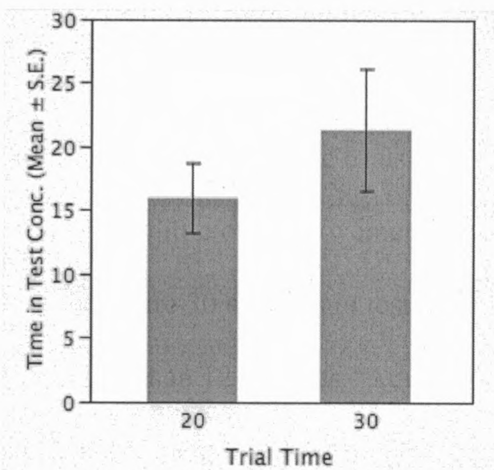

Figure 3: Mean total time in 0.5 M for wood frogs (*Rana sylvatica*) during 20 and 30-minute choice trials.

Rate of mass change

Percent mass change was calculated for the 20 and 30-minute choice trials. This was done to evaluate gain or loss of water due to osmotic flux in increasing salinities. It was expected that the longer the time spent in high salt concentrations, the more mass would be lost. There was a greater rate of mass loss the more time the frog spent in 0.5 M NaCl (Figure 4). As expected there was a high percent mass loss found in the frogs that spent the most time in the test solutions.

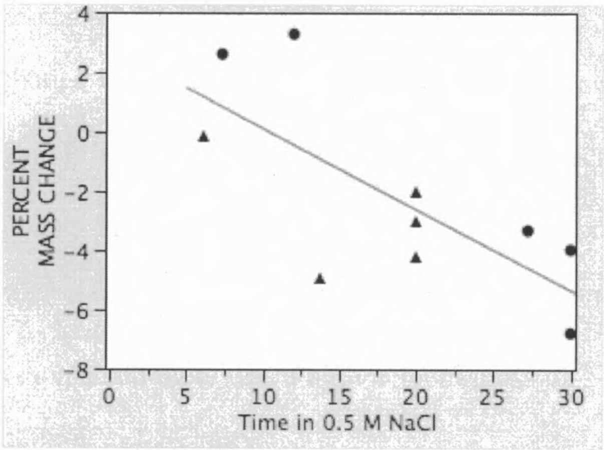

Figure 4: Individual percent mass change for 20-minute (▲) and 30-minute (●) choice trials (0.5 M NaCl vs. aged tap) was compared to linear regression for wood frogs (*Rana sylvatica*).

Effect of concentration on rate of mass change was evaluated for all timed trials (Figure 5). For green frogs, NaCl concentration has no effect on mass change in the 10-minute trials (P = 0.22) (Figure 5A). However, there may have been no effect seen because they spent less time in the higher concentrations, and got out of the test solution before it had any effect on mass change.

For wood frogs (Figure 5B), there was a significant effect of concentration; the higher the concentration, the more mass they lost over time (P = 0.049). Since many stayed in for the full 10-minutes, there was a significant change in their mass.

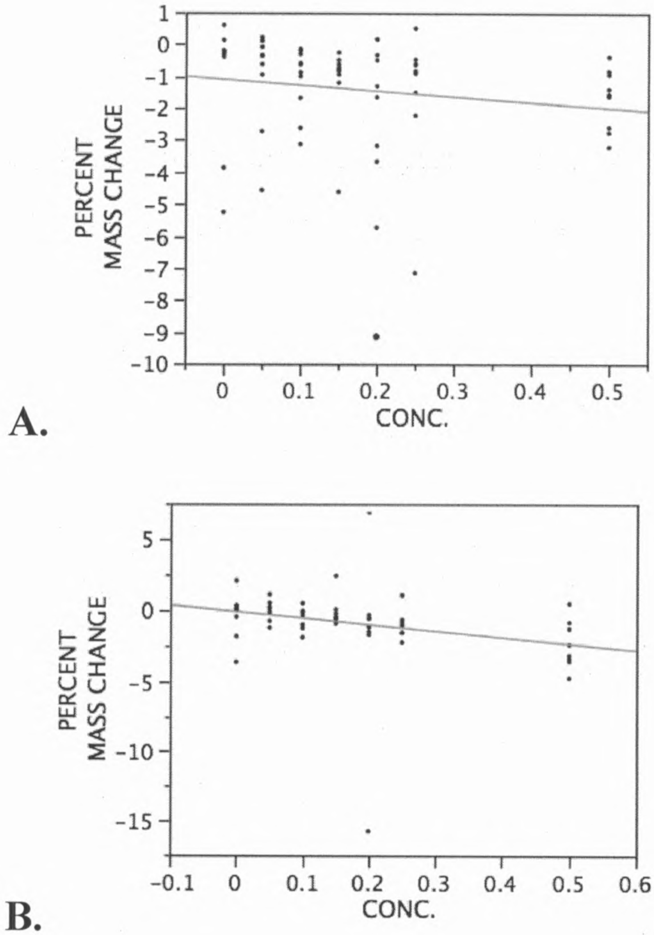

Figure 5: Percent mass loss during 10-minute choice trials. (A) Green frogs, no significant results; likely due to increasing time spent in test concentrations. (B) Wood frogs show significant mass loss with increasing concentrations.

Movement and Escape Attempts

Movements and escape attempts were noted during all timed choice trials. Average movement and escape attempts for the green frogs are 2.0 and 3.0 times in 10 minutes respectively, and 1.5 and 0.50 times in 10 minutes for the wood frogs, respectively (Figure 6). The green frogs were overall more active than the wood frogs in both movement and escape attempts.

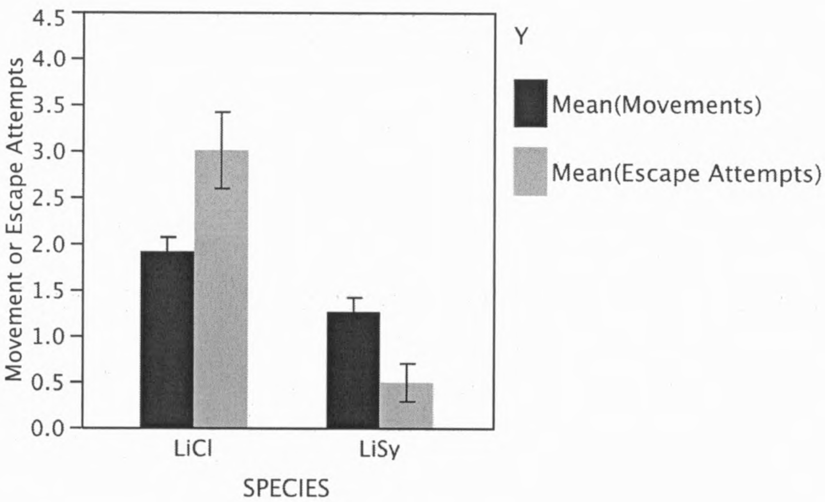

Figure 6: Mean number of movements and mean escape attempts during 10-minute choice trials for wood frogs (LiSy) and green frogs (LiCl).

Discussion

Previous research from Sanzo and Hecnar (2006) and Karraker and Ruthig (2009) has showed high salinities are detrimental to the frogs during their breeding seasons in relation to their future offspring. Karraker and Ruthig (2009) saw noticeable changes in the nervous system, mortality, altered behavior, and compromised growth and development in larval amphibians. With this study being one of the first experiments on adult wood frogs and green frogs, we now have more insight on possible problems and causes for their decline.

The species showed very different responses to varying NaCl concentrations. The constant movement by green frogs, even at low concentrations, led them to spend little time in the test concentrations even at low concentrations. Of the 70 trials with the green frogs, only four times did the subject stay in the test solution for its entirety. Most of the movements were either position changes, escape attempts (Figure 6), or active movement. Theoretically, since they were so active, they should have spent 50% of their time in each tray, however they did show a preference for the control treatment because their average time spent in 0 M was 6.33 minutes. The green frogs do show a slight aversion to increasing NaCl concentrations (Figure 1A). However, they were constantly moving between trays for the majority of the 10 minutes. This shows that they may show an aversion to increasing salinities, however more research is needed to demonstrate a more conclusive and significant aversion to increasing environmental salinity. Overall, results showed that green frogs are more active than wood frogs but still show a slight aversion to increasing NaCl salinities.

Green frogs were more active under all test conditions. Further research and possibly different research strategies may need to be employed to produce more accurate results. A tolerance threshold in green frogs (EC_{50} of 0.417 M) was determined in this experiment, but the 95% confidence interval was very broad. Although salt concentration significantly affected aversion, the level of aversion seen is relatively low. A solution that is 0.417 M NaCl is very hyperosmotic to the expected osmotic concentration of a typical amphibian, and yet the green frogs are tolerant of solutions in this range. This could be due to adaptation or acclimation to higher NaCl levels in the environment.

Osmoregulation is extremely important in keeping the body's salt concentrations balanced. As with any type of salt, altering the osmotic balance between organisms and its environment has negative effects on that organism (Findlay and Kelly 2010). As expected, the longer the frogs sat in higher NaCl concentrations, the more mass was lost due to osmotic water loss.

Sodium chloride levels were not tested from the sites where the frogs were caught. If the levels are higher than normal, the green frogs may have already adapted or acclimated to these levels, so when the choice trials were performed, they already had higher tolerance. However, a larger sample size and intermediate concentrations (0.30-0.45 M) may be able to give us more conclusive results.

The 10-minute wood frog choice trials showed very different results. Of the 56 trials, only seven did not spend the entire time in the test solution. Also, there was very little to no movement in almost every single trial. There was no significant response to increasing salinities (Figure 1B). This difference between species supports the hypothesis that the more terrestrial frogs show a lower aversion to increasing environmental salinities.

After observing no aversion to increasing salinities in 10-minutes, 20-minute and 30-minute trials were then performed to see if longer choice trials would induce a response. The 20-minute trials resulted in a mean total time in of 16 minutes, while the 30-minute choice trials showed a mean total time in of 23 minutes. This still showed little to no aversion to increasing salinities. The wood frogs have a high salinity threshold and therefore may breed in waters with a high NaCl concentration either way.

Finding out that wood frogs show little aversion to high NaCl concentrations gives us a better understanding of some of the possible causes of population declines. Sanzo and Hecnar (2006) studied the toxic effects that NaCl has on wood frog tadpoles. They found negative effects of decreased survivorship, weight and activity levels, early metamorphosis, and developmental abnormalities as concentrations increased (Sanzo and Hecnar 2006). This raises more questions concerning adult insensitivity to increasing concentrations and the potential effects on their offspring. The freeze tolerance mechanism may also play a role in their high threshold, but further research is needed.

Species that cannot regulate their body temperature in cold weather have adapted two mechanisms for survival: freeze avoidance in which body fluids are maintained in a super-cooled state, and freeze tolerance, in which extracellular body fluids endure freezing (Voituran, Barre, Ramlov et al. 2009). Wood frogs are among the species that uses freeze tolerance as their mechanism for survival because they do not possess the ability to burrow to deeper levels below the soil to avoid freezing temperatures. When the extracellular fluid freezes, physiological consequences of freezing, such as physical damage to tissues, reductions in cell volume, increases in intracellular osmolality, excess water loss, and overall problems with vital signs are drastically decreased (McNally, Sturgeon, and Storey, 2002). The wood frog has developed the freeze tolerance mechanism to cope with freezing temperatures at shallow levels below the surface. Wood frogs are capable of turning 70% of their total body water into extracellular ice in order to avoid freezing to death (McNally, Sturgeon, and Storey, 2002). The freeze tolerance mechanism involves the accumulation of osmolytes in extracellular fluid, and wood frogs therefore already have a high tolerance of increased plasma osmolarity (Muir, Costanzo and Lee, 2007).

Since wood frogs are capable of freeze tolerance and capable of surviving daily bodily water loss, how do they respond to high salt concentrations? Results showed no aversion to increasing NaCl concentrations in 10-minute trials, and a very slight aversion in 20-minute and 30-minute trials. We also found that the more time they spent in high concentrations, the more mass they lost. Taking this into account, these frogs are either unaware of the increasing concentrations and therefore do not respond or they are sensing the increased salt and show no response due to their freeze tolerance-related high tolerance of dehydration.

Further research needs to be performed to support the belief that the more terrestrial the frog, the greater the tolerance of increasing environmental salinity. A larger sample size would improve the results; a sample size of five is relatively low. Other possible experiments include testing intermediate salinities to find a more exact threshold for aversion and examination of other species on the aquatic-terrestrial spectrum.

In summary, green frogs showed some aversion to increasing environmental salinities while wood frogs showed little to no aversion. More research needs to be done to refine the threshold for green frogs and possibly conduct longer-term trials on wood frogs to determine if they exhibit a threshold following greater salt exposure. The reasons for the wood frogs exhibiting little to no aversion are still unknown, but hopefully this research has given us more insight into the drawbacks of environmental deterrents such as de-icing road salts.

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