EFFECTS OF TEMPERATURE AND NITROGENOUS WASTES ON SURVIVAL AND GROWTH OF THE BARTON SPRINGS SALAMANDER *EURYCEA SOSORUM*

by

Justin C. Crow, B.S.

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Committee Members:

Joseph R. Tomasso, Chair

Michael R. J. Forstner

Kenneth G. Ostrand

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ABSTRACT

The Barton Springs Salamander (BSS), *Eurycea sosorum*, is a federally endangered obligate aquatic salamander found only in a few spring outflows located in a highly urbanized recreational area of Austin, Texas. The purpose of this study was to gain essential information regarding the physiological response of the BSS to thermal manipulations and three common aquatic nitrogenous toxins (ammonia, nitrite, and nitrate). All salamanders used in this study were produced at the San Marcos Aquatic Resource Center (U.S. Fish and Wildlife Service) as part of a captive breeding program. To examine thermal stressors, salamanders were subjected to a temperature increase of 0.5°C per day until a loss-of-righting response (LRR) was observed. Additionally, salamander growth was assessed following a 69 day trial in which young salamanders were reared at five different temperature treatments (nominal 15, 18, 21, 24 and 27°C). The cumulative ET50 of the LRR for the combined replicates observed in the BSS was 32.6 ± 0.24°C (mean ± SD). The optimal temperature for growth of the BSS for total length was estimated to be 18.3°C resulting in a 59.7 ± 21.09% increase in total length. To investigate the effects of nitrogenous wastes on the BSS, ninety-six hour median-lethal concentration (96-hour LC50) trials were conducted for un-ionized ammonia-N (UIA-N), nitrite-N, and nitrate-N. The 96-hour LC50 of UIA-N, nitrite-N, and nitrate-N to the BSS was 2.1 ± 0.19 mg/L, 27.7 ± 0.72 mg/L, and 851.1 ± 49.21 mg/L, respectively. These results will aid in the conservation, management, and ongoing efforts to culture the BSS in captivity.

PREFACE

Public awareness of the recent decline in amphibians has increased considerably since this phenomenon was first acknowledged at a National Research Council workshop in the early 1990's (Wake 1991). Around the same time, public attention in Central Texas was focused on the Barton Springs Salamander (BSS), *Eurycea sosorum*, with habitat located in the rapidly developing area of Austin, Texas. Politicians, citizens, and developers fervently deliberated proposed conservation measures for the BSS, and ultimately the BSS was granted endangered status by the U. S. Fish and Wildlife Service in 1997 (USFWS 1997). In order to develop and implement effective conservation measures, an understanding of the environmental requirements of the organism of concern is necessary. One of the most influential environmental factors affecting the BSS is temperature, which remains almost constant in its stenothermal habitat. However, information concerning the physiological response of the BSS to thermal changes is lacking. Further, studies have been conducted on the physiological and developmental effects of several insecticides on the BSS (USEPA 2007a and b), yet information concerning the physiological response of the BSS to common aquatic nitrogenous toxins present in its environment remain unknown. Thus, the following study sought to examine the physiological tolerances of the BSS to thermal manipulations (Chapter 1) and three common aquatic nitrogenous toxins (Chapter 2).

CHAPTER I

EFFECTS OF MULTIPLE THERMAL STRESSORS ON THE BARTON SPRINGS SALAMANDER (*EURYCEA SOSORUM*)

Introduction

The Barton Springs Salamander (BSS), *Eurycea sosorum,* (Chippendale *et al*. 1993) is a state and federally listed endangered plethodontid salamander found in the Barton Springs segment of the Edwards Aquifer. The known geographic range of this species is restricted to four spring openings, collectively known as Barton Springs, in the Barton Creek drainage located near downtown Austin, Texas (Petranka 1998). This species occupies a stenothermal habitat with perennial spring flow and substrates consisting of rock, cobble, gravel, and various macrophytes. Given the adaptation of the BSS to a mostly stable thermal environment, it has been reasonably concluded that the BSS is at minimum partially dependent on consistent water temperatures (USFWS 2005).

Temperature is one of the most important abiotic factors affecting amphibians and influences physiological functions, such as metabolic rate, gas exchange, reproduction, development and growth (Hillman *et al*. 2009). For stenotherms, maintenance of a narrow range of environmental temperatures is necessary for optimal physiological function. Numerous studies have demonstrated the detrimental effects to salamanders caused by temperature extremes (Zweifel 1957; Hutchison 1961; Rohr and Palmer 2012). Annual mean water temperatures in Barton Springs normally range from 21 to 22°C (USFWS 2005). However, intermittent spring flow is reported to occur at Upper Barton Springs (Dries *et al*. 2013). While no thermal information is available, it is reasonable to

think that water temperatures vary more widely during the interruption of flow than during normal flow. The objectives of this study were to determine the relationship between a slow increase in temperature (mean increase of 0.5°C per day) on the loss-of-righting response reflex (LRR), and determine the effect of temperature on growth of the BSS. This information can be incorporated into the recovery plan (USFWS 2005) for this federally listed species.

Materials and Methods

Thermal manipulation experiments were conducted at the San Marcos Aquatic Resource Center (SMARC, U.S. Fish and Wildlife Service), in San Marcos, Texas. All salamanders used in this study were produced at the SMARC as part of a captive breeding program (USFWS 2005). Multiple, insulated, temperature-controlled, 1,135-L fiberglass tank systems were filled approximately half full with well water from the Edwards Aquifer to serve as thermally stable water bath reservoirs. Three 75-L experimental tanks were placed in each water bath reservoir approximately two-thirds submerged and used to hold salamanders during trials. Polyvinyl chloride (PVC) pipe halves (5 cm diameter; thermal maxima experiment) or rocks and macrophytes (optimal growth experiment) were placed in each experimental tank in order to provide shelter for the salamanders. A submersible pump was also placed in each experimental tank to provide circulation.

Thermal maxima experiment. Fifteen BSS were placed into each of three experimental tanks. The salamanders were then allowed to acclimate for 24 hours prior to treatment. Initial water temperature was 21 ± 1°C, which is similar to their natural spring

habitat and captive assurance colony holding systems. The temperature was then raised a nominal 0.5°C per day (actual mean temperature increase = 0.46°C per day, $r^2 = 0.997$) until all salamanders had exhibited a LRR. The LRR is characterized by a loss of equilibrium so that upon sinking to the bottom after a period of swimming, the salamander may rest upon its back for a time before righting itself (Zweifel 1957). The gradual increase in temperature should give the animals an opportunity to physiologically acclimate to the increasing temperature (Lowe and Vance 1955; Spotila 1972), and serve as an indicator of maximum tolerable temperature. Once the temperatures approached the salamanders suspected upper thermal limits, based on decreased feeding, hyperactivity, and thermal maxima values for salamanders obtained from literature (Zweifel 1957; Sealander and West 1969; Spotila 1972; Berkhouse and Fries 1995; Lutterschmidt and Hutchison 1997), we began monitoring the salamanders for several minutes every hour until the temperature stopped increasing, and then every three hours after that. At the first indication an individual was experiencing a LRR, that individual was removed, weighed (grams), and measured (SVL, snout to vent length; the distance from the tip of the snout to the posterior margin of the cloaca; and TL, total length; the distance from the tip of the snout to the tip of the tail). Immediately following measurements, salamanders were placed in a separate experimental tank maintained at the same experimental temperature, and allowed to cool gradually (approximately 1°C per hour) in an attempt to facilitate recovery of the salamander. Mean salamander weight in the thermal maxima experiment was 1.2 ± 0.40 g (mean ± SD), and mean SVL and TL were 37 ± 3.5 mm and 73 ± 9.4 mm, respectively.

Temperature, pH (8.0 ± 0.31), and dissolved oxygen saturation (86 ± 10.0%) were measured daily during the trial using a Hydro-tech MS5 (Hach Co., Loveland, CO). Total ammonia-nitrogen (0.3 ± 0.31 mg/L) was measured up to three times per week throughout the trial by direct Nesslerization (APHA 1989). The salamanders were fed to excess a diet of commercially produced live black worms, *Lumbriculus variegatus*, (California Blackworm Co., Fresno, CA) and brine shrimp, *Artemia salina*, (Mariculture Technologies International, Inc., Oak Hill, FL). Light cycle was maintained under natural photoperiod conditions via overhead skylights and uncovered windows.

Optimal growth experiment. Six to seven salamanders (TL range from 15 to 40 mm) were stocked into each of fifteen 75-L experimental tanks. Each experimental tank was partially submerged in one of five temperature-controlled fiberglass tank systems which served as a thermally stable water bath reservoir (three experimental tanks per water bath reservoir). Salamanders were then given 24 hours to acclimate at a water temperature of 21 ± 1°C. The temperature in each of the five water bath reservoirs was then adjusted to a nominal 15, 18, 21, 24 and 27°C at a rate of ± 1°C per day (Sadler 1979) to allow the salamanders to acclimate to treatment temperatures. Once each water bath reservoir reached the assigned temperature, salamanders were removed, weighed (grams), measured (TL in mm), and returned to their respective experimental tanks. Mean weight and TL of salamanders at the start of the trial were 0.1 ± 0.06 g and 31 ± 4.6 mm, respectively.

While in the experimental tanks, salamanders were observed at least two times per day and fed an alternating diet of *Artemia* nauplii, *Artemia salina*, (INVE Aquaculture, Inc., Salt Lake City, UT), zooplankton (pond raised at the SMARC), and

commercially produced live black worms, *Lumbriculus variegatus*, (California Blackworm Co., Fresno, CA) to excess. Temperature was monitored daily using a Hach HQ40d meter (Hach Co., Loveland, CO) and remained within a mean of 0.2 ± 0.21°C of nominal settings across temperature treatments. Dissolved oxygen saturation (92 ± 7.3%, across all treatments), and pH (8.3 ± 0.11, across all treatments) was monitored in each experimental tank daily using a Hach HQ40d meter. Total ammonia-nitrogen (0.8 ± 0.59 mg/L, across all treatments) was monitored in each experimental tank twice per week by direct Nesslerization. If total ammonia-nitrogen levels exceeded 0.5 mg/L in experimental tanks, a 40% water change was performed using fresh Edwards Aquifer well water that had been maintained at the same experimental temperature as the respective experimental tank.

Once salamanders had spent 69 days at their respective temperature treatments they were removed from their experimental tanks and individually weighed and measured. The salamanders were then returned to their respective experimental tanks and acclimated back to their natural habitat and holding temperature of 21°C, using the method of 1°C per day (Sadler 1979) as previously described.

Regression analysis was used to analyze data from both experiments, with a significance level set at a $p \leq 0.05$. The statistical software package R Studio (R Core Team 2014) was used to determine probability values. In the thermal maxima experiment linear regression was applied to each replicate ($N = 3$) and to all replicates combined. The regression of the combined replicates is shown in Figure 1. The effective temperature at which half of the salamanders displayed a LRR (ET50) was calculated for each replicate, as well as the combined replicates along with the 95% confidence interval. Linear

equations generated by each regression were used to calculate ET50 values by assuming a 50% value for the dependent variable (% LRR) and solving for the independent variable value (Temperature). For the optimal growth experiment, the mean of all logged temperature readings (N=70 temperature readings per experimental tank) obtained during the trial and mean salamander growth (% increase in weight and TL of salamanders in experimental tank) in each experimental tank were used in the polynomial regression analyses. This resulted in an *N* of three for each temperature treatment. Optimal growth temperatures and associated 95% confidence intervals were calculated for regressions. Optimal growth temperatures were estimated from the quadratic equation generated by regression curves by calculating the estimated vertex of the curve using the following equation; $\hat{x} = -\frac{b}{2a}$

Results and Discussion

Thermal maxima experiment. The first observed instance of LRR in the BSS occurred at 31.7°C and all BSS had exhibited a LRR or died by 33.5°C. Episodes of LRR were distinguished by the preliminary loss of posterior limb function, consistent with Hutchison (1961), followed by moments of disoriented swimming. Linear regression was applied to each replicate, as well as all replicates combined. However, only the regression of combined replicates ($r^2 = 0.98$, $P < 0.001$) is shown in Figure 1. The ET50 for the LRR in the BSS ranged from 32.4 ± 0.45°C (95% confidence interval of 32.17 to 32.63°C) to 32.9 ± 0.44°C (95% confidence interval of 32.67 to 33.12°C) for all three replicates. For all replicates combined the ET50 for the LRR in the BSS was of 32.6 ± 0.24°C (95% confidence interval of 32.45 to 32.75°C, Figure 1).

Our results were consistent with numerous thermal tolerance studies of plethodontids (Sealander and West 1969; Spotila 1972; Berkhouse and Fries 1995; Lutterschmidt and Hutchinson 1997), which indicate episodes of mean LRR at similar temperatures for *Plethodon ouachitae* (30.6 ± 0.25°C), *Plethodon richmondi* (31.3 ± 0.48°C) and *Desmognathus ochrophaeu* (29.2 ± 0.49°C). However, many past studies utilized heating rates of 0.5 to 1.0°C per minute. These rapid heating rates were justified as necessary to account for the rapid rate at which most reptiles absorb or lose heat (Cowles and Bogert 1944). A reduced heating rate of 0.5°C per day was chosen for the current study to allow the animals to physiologically acclimate to changing temperatures, perhaps giving a better estimate of their upper thermal limits. Moreover, these are more likely to approximate conditions in the field in the absence of spring flow or during reduced spring flows.

Optimal growth experiment. A total of 3 BSS died during the temperature treatment exposures (97% survival). Initial weights and initial TL were not significantly different among treatments at the beginning of the experiment ($P = 0.265$ and $P = 0.464$, respectively). Temperature had a significant effect on the percent increase in TL of the BSS ($P = 0.018$, Fig 2 A). However, when measured as percent increase in weight of the BSS, temperature did not have a significant effect ($P = 0.175$, Fig 2 B). The optimal temperature for growth of the BSS for increase in TL was estimated to be 18.3°C resulting in a 59.7% increase in TL (95% confidence interval of 27.96 to 91.49%). Further, the percent increase in TL at all tested temperatures, except 27°C, fall within the 95% confidence interval for the percent increase in TL among optimal temperature estimates. The optimal temperature for growth of the BSS for increase in weight was not

estimated due to the lack of a significant treatment effect. Qualitatively, a trend toward reduced increases in TL began to occur just beyond 21°C, but as only the 27°C treatment was significantly different, a range of the tested temperatures (15-24°C) accommodates similar growth. The least overall increases in both weight and TL was observed in the 27°C treatment (actual mean temperature = 27.7°C), which is well outside the normal thermal range currently encountered in the BSS habitat.

The optimal growth temperature estimate derived from the intercept of our model is slightly below the lower end of the temperature range typically occurring in BSS surface habitat (21 to 22°C). However, as none of the treatments in the range of 15-24°C were significantly different, we can state that the surface habitat temperatures known for the BSS are within the range of temperatures bound by our confidence interval estimates for growth by TL. Our results were consistent with other studies, which indicate that within the temperature range normally encountered by an organism, a decrease in rearing temperatures causes a majority of ectotherms to attain larger sizes at a given developmental stage (Atkinson 1994). Additionally, it has been shown that reduced growth occurs with increased rearing temperatures in ectotherms (Atkinson 1995). However, this reduced growth generally results when an ectotherm is outside of its optimal temperature range for performance (Huey and Kingsolver 1989). Temperature essentially controls the metabolic rate and metabolic scope of such organisms. Metabolic scope is described as the ratio of the maximum sustained metabolic rate to the standard metabolic rate (SMR) for poikilotherms (Randall *et al*. 1997). At SMR for poikilotherms, metabolic oxygen demand generally increases exponentially with temperature. However, the physiological capacity to supply this oxygen increases sigmoidally with increasing

temperatures (Neill and Bryan 1991). Thus, the temperature of maximum metabolic scope is the optimal temperature for an organism. As temperatures increase beyond an organism's optimal temperature, metabolic scope begins to decrease, subsequently affecting the physiology and growth of an organism. The results from this study would support an optimal temperature range for optimal growth of *Eurycea sosorum* that is much broader than is often speculated (USFWS 2005; Pierce and Wall 2011).

Given the ongoing efforts to culture the BSS in captivity (Chamberlain and O'Donnell 2003; USFWS 2005), these results will provide basic thermal information for the culture of the BSS for captive assurance colonies. Further, our results provide vital thermal tolerance information for the BSS, which may be of value to habitat managers as they try to estimate the impacts of changing habitat temperatures and conditions on the BSS (Turner 2004). Temperatures exceeding 26°C in Old Mill Spring (Dries *et al*. 2013), a known spring habitat for the BSS, have been documented. In addition, given the previously speculated thermal sensitivity of other central Texas *Eurycea* (Sweet 1982; Pierce and Wall 2011), our results will aid in the assessment of the potential impacts of climate change in the Edwards Plateau region (Loaiciga *et al*. 2000) on habitat temperatures of these species. For example, temperatures up to and exceeding 30°C have been documented (Bowles *et al*. 2006) in a known habitat of the Jollyville Plateau Salamander, *Eurycea tonkawae,* a federally listed threatened (USFWS 2013) species found along the Jollyville segment of the Edwards Plateau. Our data would support this temperature nearing the limits of the salamander's thermal maxima, but clearly beyond the temperatures supporting optimal growth. With the potential for continued increases in habitat temperatures for the BSS, as well as other central Texas *Eurycea*, an

understanding of their thermal requirements will be necessary for their continued conservation.

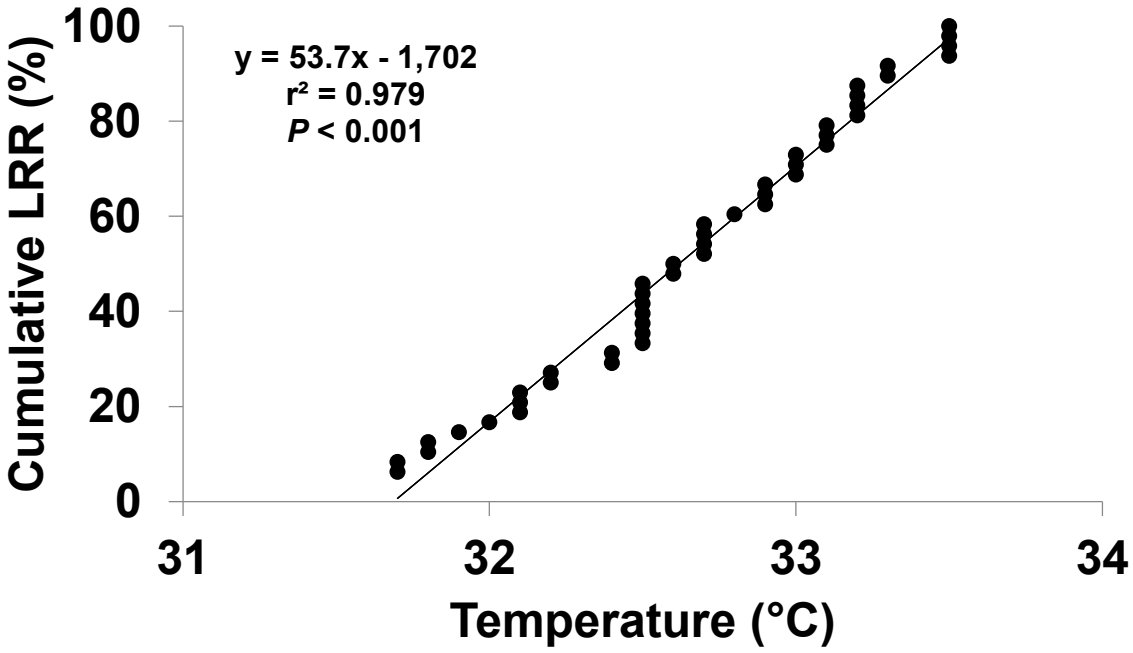

Figure 1. Cumulative Loss of Righting Response (%), for combined replicates, of the BSS in the course of daily temperature increases of 0.45°C (initial temperature = 20.8°C).

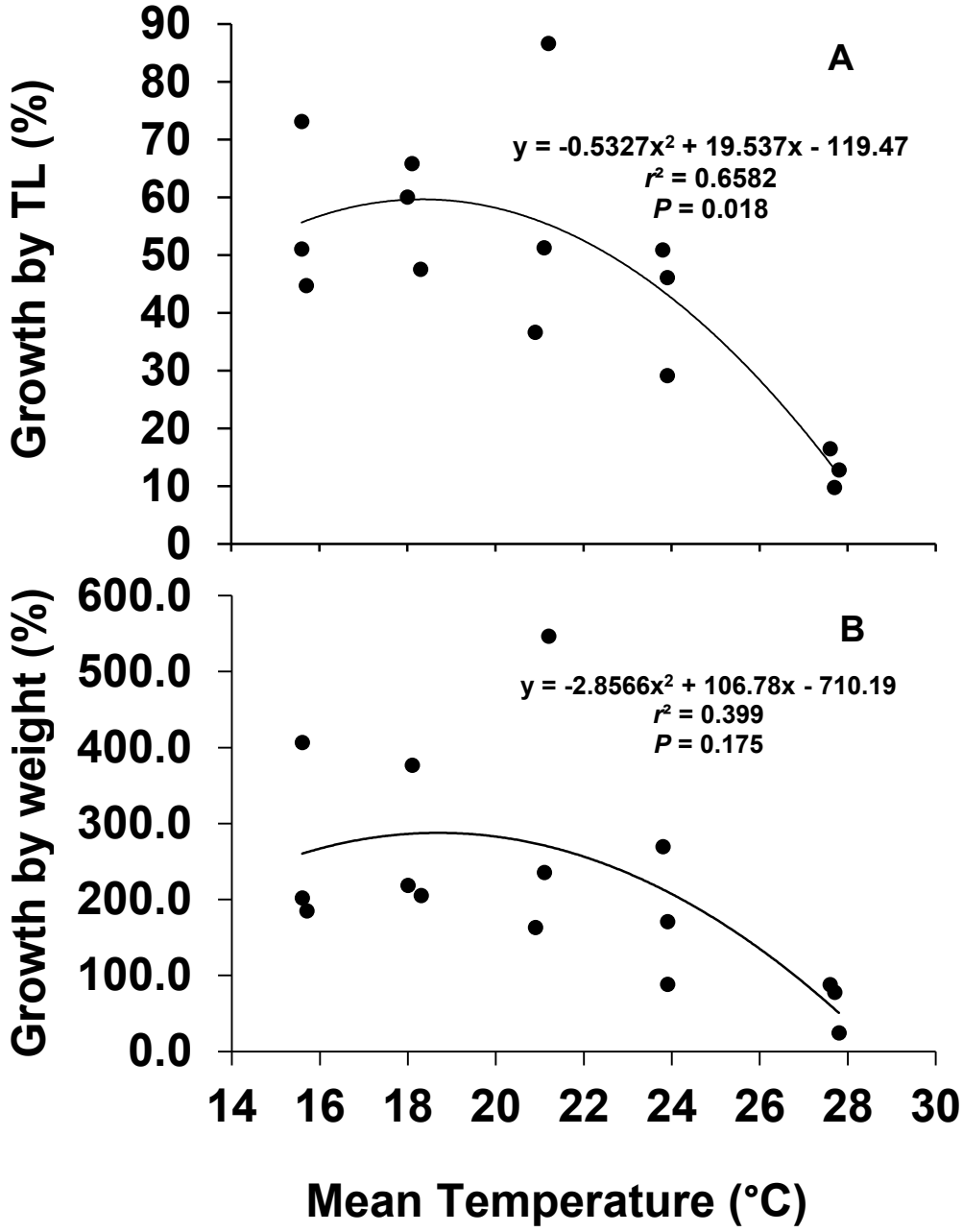

Figure 2. Relationship between mean temperature (°C) and growth by percent total length (A) and percent weight (B) of the BSS after a 69 day exposure to nominal temperatures of 15, 18, 21, 24 and 27°C. Growth by TL (A) was equivalent at all tested temperatures, except 27°C, where a significant decline ($P = 0.005$) in growth occurred**.**

CHAPTER II

ACUTE TOXICITY OF AMMONIA, NITRITE, AND NITRATE IN THE BARTON SPRINGS SALAMANDER (*EURYCEA SOSORUM*)

Introduction

The federally endangered Barton Springs Salamander (BSS), *Eurycea sosorum,* occurs in a few spring outflows, collectively known as Barton Springs, emanating from the Barton Springs Segment of the Edwards Aquifer (Green *et al*. 2014). This spring system is located in a rapidly growing, highly urbanized area near downtown Austin, Texas. Barton Springs also serves as an important recreational resource that receives around 350,000 paid visits per year (Smith *et al*. 2001). Highly urbanized environments, such as this, often produce storm water runoff with elevated levels of pollutants (Booth and Reinelt 1993). Analysis of long-term water quality data (1975 to 1999) from Barton Springs indicates increases in sulfates, total organic carbon, and specific conductance, which are presumed to be related to increased urbanization (Turner 2005). A recent study indicates that nitrate plus nitrite concentrations at Barton Springs were consistently greater than the national background concentration for groundwater and were positively correlated with spring discharge (Mahler *et al*. 2011). Given current pressures and projected increases in urbanization, it is reasonable to anticipate an increase in contaminant loads occurring in BSS habitat.

The objectives of this study were to determine the acute toxicity of three common aquatic nitrogenous toxins, (ammonia, nitrite, and nitrate) to the BSS. The results of this study were compared with other aquatic species to determine if current water quality

criteria are adequate to protect the BSS. Additionally, the following study establishes a benchmark for the tolerance of the BSS to these three toxins.

Materials and Methods

A total of 96 adult BSS were obtained from the San Marcos Aquatic Resource Center (SMARC, U.S. Fish and Wildlife Service) in San Marcos, Texas and transferred to Texas State University in San Marcos, Texas. Salamanders used in this study ranged in size from 45 to 80 mm (mean ± SD = 65 ± 12.8 mm) and were produced at the SMARC as part of a captive breeding program (USFWS 2005). All salamanders were held in the BSS captive breeding refugium at the SMARC until 24 hours prior to exposure. At which time eight individuals per toxicity trial were transported to Texas State University.

Toxicity tests were conducted at Texas State University. A single 720-L fiberglass tank served as a thermally stable water bath reservoir by receiving a constant flow of Edwards Aquifer water adequate to maintain a temperature of approximately 22°C. Ninety-six hour median-lethal concentration (96-hour LC50) trials were conducted in eight 30-L experimental tanks that were partially submerged in the reservoir to serve as water baths. One salamander was placed into each of the eight experimental tanks so that there were a total of eight independent observations for each trial. Several sterile rocks were placed into each experimental tank to provide habitat. A submersible pump was placed into each experimental tank to provide water flow. Each experimental tank contained 10-L of Edwards Aquifer water (14 mg sodium/L, 1 mg potassium/L, 60 mg calcium/L, 20 mg magnesium/L, 23 mg chloride/L, 30 mg sulfate/L) in order to reduce chemical usage and standardize dosage calculations.

Salamanders were stocked into experimental tanks 24 hours before toxin exposure. A modified version of the USEPA toxicity testing protocol (USEPA 2002) was used. The modification was a reduction of the number of animals from 15 to 1 per replicate, reducing by approximately 93% the number of animals needed for the study.

Initial exposure concentrations were estimated from literature (Camargo *et al*. 2005; Griffis-Kyle 2007; USEPA 2010; USEPA 2013) and concentrations were increased by a geometric progression factor of two. For each trial eight animals were exposed at a time (including a control which received no toxin). Based on the results of each trial, more animals were exposed until we had four concentrations, near the anticipated LC50, to each of which five animals had been exposed. Thus, the ammonia, nitrite, and nitrate toxicity trials were replicated five times each. Dead salamanders (total N = 85) were removed from aquaria, measured, and preserved (95% ethanol). A summary of toxin concentrations can be found in Table 1.

Salamanders were fed during the exposure an alternating diet of live brine shrimp, *Artemia salina*, (Mariculture Technologies International, Inc., Oak Hill, FL) and commercially produced live black worms, *Lumbriculus variegatus*, (California Blackworm Co., Fresno, CA). Temperature (22 ± 1.0°C for all trials) and dissolved oxygen saturation (90 ± 9.0% for all trials) were measured daily during exposure using a YSI 2030 meter (YSI, Inc., Yellow Springs, Ohio). At the end of each exposure, pH (8.3 ± 0.31 for all trials) was measured (Accumet AB15 plus pH meter; Thermo Fisher Scientific Inc., Walthma, MA). At the end of each exposure, total ammonium-nitrogen, nitrite-nitrogen, or nitrate-nitrogen levels were measured, depending on exposure treatment. Total ammonia-nitrogen was determined by direct Nesselarization (APHA

1989). Nitrite-nitrogen and nitrate-nitrogen were determined spectrophotometrically using a GENESYS 20 spectrophotometer (Thermo Fisher Scientific Inc., Walthma, MA) with the reagents NitriVer® 2 nitrite reagent and NitraVer® 5 nitrate reagent (Hach Company, Loveland, CO), respectively. Nominal concentrations of ammonia, nitrite, and nitrate were developed by the addition of ammonium chloride, sodium nitrite, and sodium nitrate (Thermo Fisher Scientific Inc.), respectively. Un-ionized ammonia-nitrogen (UIA-N) concentrations were calculated from tables in standard methods for the examination of water and wastewater (APHA 1989). At the end of the toxicity tests, the mean percent nominal concentrations of UIA-N, nitrite-N, and nitrate-N were 90 ± 5.8%, 92 ± 4.6% and 101 ± 13.6%, respectively. Following each toxicity trial, each experimental tank was drained, rinsed, dried, and refilled with fresh aquifer water. The laboratory light cycle was maintained on a 12:12 light dark photoperiod.

Data were analyzed using the Spearman-Karber Method (Finney 1978), which is a non-parametric procedure used to estimate median-lethal dose/concentration values and associated 95% confidence intervals (USEPA 2002). This method estimates the mean of the distribution of the logarithm of the tolerance. This non-parametric procedure was chosen due to the violation of the assumptions of normality and homoscedasticity caused by a binary response variable. The 96-hour time course was retained for this study because the toxicity-testing database consists of mostly 96-hour studies, allowing better integration of our results with the literature.

Results and Discussion

The 96-hour LC50 of UIA-N to the BSS was 2.1 ± 0.19 mg UIA-N/L (95% confidence interval of 1.6 to 2.9 mg UIA-N/L). The first mortality was observed at 2 mg UIA-N/L (Table 1). All mortalities occurred in the first 24 hours. The 96-hour LC50 of UIA-N to the BSS is similar to that of the Leopard frog, *Rana pipiens*, when exposed as embryos (1.2 mg UIA-N/L, Diamond *et al*. 1993) and eggs (2.2 mg UIA-N/L, Jofre and Karasov 1999). The acute toxicity of the BSS to UIA-N is generally higher than that of many freshwater fishes (Haywood 1983; Tomasso 1994; USEPA 2013).

The 96-hour LC50 for nitrite-N was 27.7 ± 0.72 mg NO_2-N/L (95% confidence interval of 21.8 to 35.1 mg NO_2-N/L). The first mortality was observed at 32 mg NO_2-N/L. The BSS shows greater resistance to nitrite-N than small-mouth salamander *Ambystoma texanum* larvae, which have a 96-hour LC50 of 1.09 mg NO_2-N/L (Huey and Beitinger 1980). However, the tolerance of the BSS to nitrite-N appears average when compared to freshwater fish species, with the LC50 of nitrite-N to the BSS falling midway between most LC50 values of many freshwater fishes (Russo and Thurston 1977; Tomasso 1986; Lewis and Morris 1986).

The 96-hour LC50 for nitrate-N was 851.1 ± 49.21 mg NO_3-N/L (95% confidence interval of 562.7 to 1,287.4 mg NO_3-N/L). The first mortality was observed at 500 mg NO_3-N/L. The 96-hour LC50 of nitrate-N to the BSS is similar to that of the washboard mussel *Megalonaias nervosa* (937 mg NO_3-N/L, USEPA 2010). The BSS appears to have a higher tolerance for nitrate-N than Pacific tree frog *Pseudacris regilla* embryos (643 mg NO_3-N/L, Schuytema and Nebeker 1999a). However, when the Pacific tree frog is exposed to nitrate-N at the larval stage, the BSS has a considerably lower threshold for

nitrate-N than the Pacific tree frog (1,749.8 mg NO_3-N/L, Schuytema and Nebeker 1999b). A considerable amount of literature has tested the acute effects of nitrate-N on amphibian eggs and/or larvae (Laposata and Dunson 1998; Camargo *et al*. 2005; Meredith and Whiteman 2008). However, it is difficult to draw a direct comparison to our results, which are based on adult organisms.

The toxicity of nitrogenous wastes can vary based on water quality characteristics, such as temperature, pH, and chloride (Tomasso *et al*. 1980; Williams and Eddy 1986; Russo and Thurston 1991; Tomasso 1994). The effects of chemical constituents in water must be considered when conducting and interpreting toxicity tests. Lack of consideration of the variation in water chemistry between test locations may result in disparities in toxicity estimates across species and within species, resulting in wide-ranging and confusing LC50 values (Lewis and Morris 1986).

The United States Environmental Protection Agency (USEPA) establishes National Ambient Water Quality Criteria (AWQC) under the Clean Water Act (Clean Water Act 1972). These criteria serve as recommendations for defining ambient water concentrations that will protect against adverse ecological effects to aquatic life as a result of pollutants (USEPA 2013). Documents are available evaluating the toxicity of specific chemicals, including ammonia (USEPA 2013), while nutrient criteria documents are available for nitrite and nitrate (USEPA 2001). As data become available, AWQC are refined and adjusted. In 1999, the USEPA updated the AWQC to include and account for the effects of temperature and pH on the toxicity of ammonia (USEPA 1999), adjusting all subsequent AWQC documents. Additionally, as data become available, more species will be added and subsequently used to estimate water quality criteria. Currently, out of

the 100 species that were included in the 2013 AWQC document for ammonia, only four were amphibians, all of which were anurans. This lack of toxicity data available for amphibian species is concerning. Without sufficient knowledge of an organism's environmental requirements and tolerances, governmental agencies may fail to provide adequate protections for sensitive organisms.

Our results demonstrate that current USEPA water quality criteria recommendations for acute exposures to total ammonia-nitrogen, 17 mg/L, or UIA-N, 0.066 mg UIA-N/L (USEPA 2013), are adequate to protect the BSS. Further, the USEPA ranks the sensitivity of the organisms to total ammonia-nitrogen based on a ranked genus mean acute value (GMAV). These values are then ordered by sensitivity, with the most sensitive organisms given the lowest rankings. Based on our results, the BSS would rank 44 out of 69 genera with a ranked GMAV of 156.9 mg total ammonia-nitrogen/L (adjusted for a pH = 7.0) in the USEPA AWQC for ammonia (USEPA 2013). Our results also demonstrate that current water quality criteria recommendations for both nitrite and nitrate measured as the sum of nitrite and nitrate, 0.05 mg/L for Ecoregion 32 (USEPA 2001), are adequate to protect the BSS.

Table 1. Results of 96-hour LC-50 toxicity trials on the BSS

N	Dosage (mg/L)	% Survival
	UIA-N	
1	0.25	100
1	0.5	100
5	1	100
5	2	60
5	4	0
5	8	0
1	16	0
	Nitrite-N	
1	0.5	100
1	1	100
1	2	100
1	4	100
6	8	100
6	16	100
7	32	29
6	64	0
	Nitrate-N	
2	1	100
2	10	100
6	100	100
7	500	86
6	1000	50
5	2000	0
4	4000	0

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