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# Stress responses to salinity in a native fresh water snake (Nerodia rhombifer)

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# STRESS RESPONSES TO SALINITY IN A NATIVE FRESH WATER SNAKE (*NERODIA RHOMBIFER*)

A Thesis

by

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STRESS RESPONSES TO SALINITY IN A NATIVE

FRESH WATER SNAKE

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# ABSTRACT

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Salinity stress at 0 ppt, 9 ppt, 18 ppt, and 27 ppt was investigated in *Nerodia rhombifer*, a fresh water snake. The purpose of this study was to establish if exposure to salinity could elicit a hormonal response in the form of corticosterone. Salinity treatment 27 ppt was lethal for 50% of the snakes. Factors such as population and gender differences were not significant, $p$=0.748 and $p$=0.135, respectively. Exposure to the different salinities did not significantly affect the overall mass of the animal, $p$=0.951. A significant increase in circulating corticosterone was noted in salinity treatments 0 ppt and 18 ppt, $p$=0.038. The data collected from this study, can be useful in explaining the distributional limits for *N. rhombifer* along the Rio Grande and in other riverine systems.

# DEDICATION

I would like to thank my extended and immediate family for all of their help and patience in aiding the completion of this thesis. A special thanks to my aunt in-law, Ampelia Garcia, and my sister, Marilyn Cordoba, for babysitting my son while I finished writing. I also want to thank my mom, Maria Cordoba, my dad, Carlos Cordoba, my sister, Carla Cordoba, and my husband, Alberto Gonzalez for encouraging me to fulfill my dream. The study in this thesis was the work of four long years, the last in which my son was born and to him, Saul Giovany Gonzalez, I dedicate this body of work.

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# CHAPTER 1

## INTRODUCTION

Museum records of *Nerodia rhombifer* dating back to 1900 show collections as far upstream the Rio Grande as the Pecos River, but records in 1969 show the distribution starting further downstream at the Devil's River and continuing downstream to Brownsville (Conant, 1969). Over the past century the Rio Grande's physical environment has changed mainly due to water withdrawals, damming and salinity intrusions from the Pecos River (Coastal Impact Monitoring Program, 1995). High levels of salinity along the river serve as ecological barriers for native fauna that are salt intolerant (Robinson, 1959). These changes have drastically affected freshwater species that previously lived in the Lower Rio Grande Valley region (Edwards and Contreras-Balderas, 1991). The potential exposure to salinity makes studying salinity stress in the species *N. rhombifer* extremely interesting.

Man-made canals that serve as highways to artificial ponds and lakes are connected to the Rio Grande by an irrigation system. This expands the home range of *N. rhombifer* far inland. *Nerodia rhombifer* is known as one of the heaviest snakes and largest in length of the state of Texas (Dixon, 1984). The species is widely distributed across Texas' canals, lakes, ponds, and rivers, which is noteworthy since many of these are ephemeral and/or can vary in salinity during the dry season. These snakes are semiaquatic and highly active at night. They seldom stray more than 2 m away from a

water source and are almost exclusively piscivorous, hence the dependency upon water (Gibbons and Dorcas, 2004). They are an ovoviviparous species giving birth to litters ranging from six to sixty-two offspring, which for the most part makes them abundant for studying (Gibbons and Dorcas, 2004). Many physiological studies have been performed on *N. rhombifer* mainly because of their availability (Werler and Dixon, 2002). In general, *N. rhombifer* is an excellent animal model to test the effects of salinity exposure.

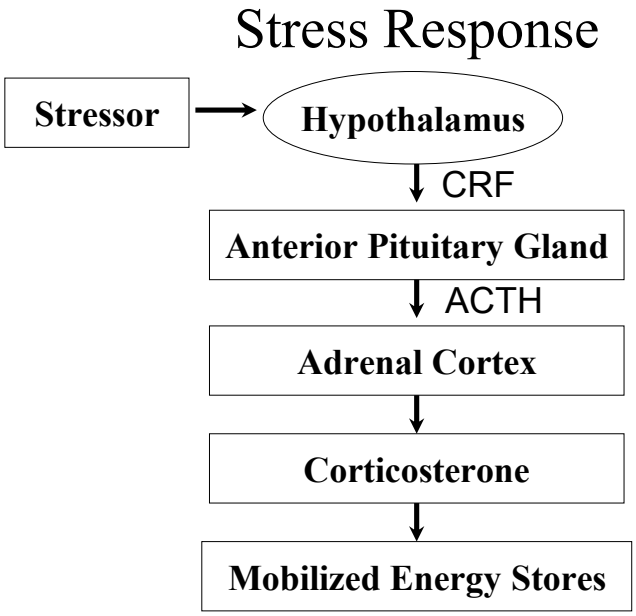
Many snakes (including *N. rhombifer*) can tolerate some salinity exposure, even though they do not have specialized physiological and morphological mechanisms (e.g., salt glands) to secrete excess salt (Dunson and Mazzotti, 1989). Abiotic factors, such as salinity, do influence the biochemical and physiological functions of aquatic organisms (Dunson and Travis, 1994). Although *N. rhombifer* is not constrained to just aquatic environments, its source of food and water are all tied to these environments. Ingesting large amounts of salt in its food or water source can potentially cause an imbalance in their internal osmolality (Rankin and Davenport, 1981).

Osmolality is a measure of the amount of dissolved solutes (e.g., sodium, chloride, potassium, urea, and glucose) present in the blood (Rankin and Davenport, 1981). The osmolality of an organism is maintained within a certain range; for example in *N. rhombifer* the osmolality ranges from 297 to 336 mOsm/kg $H_2O$ (McDaniel et al., 1984). In general, plasma osmolality is tightly regulated because it affects cellular function, secretory activity, and membrane permeability (DuRant et. al., 2008)

The ingestion of salty water from the environment can cause a rise in plasma osmolality, which triggers the release of antidiuretic hormone and activates thirst receptors (Peaker and Linzell, 1975; Bentley, 1976; Rankin and Davenport; 1981).

Consequently, tolerance to some environmental salinity may also cause the animal to experience an inordinate amount of stress measurable in their release of hormones (Peaker and Linzel, 1975; Bentley, 1976; Rankin and Davenport, 1981). The main stress hormone in reptiles is corticosterone and thus, corticosterone is part of the stress pathway (Figure 1) (Peaker and Linzel, 1975; Bentley, 1976; Rankin and Davenport, 1981).

Figure 1. The stress response pathway. The arrows show the direction of the pathway and in turn the stimulation of each section (Wilmer, Stone, and Johnston, 2004). CRF stand for corticotropin releasing factor and ACTH is the adrenocorticotropic hormone.




The stress pathway's activation starts with a stressor that stimulates the hypothalamus to release corticotropic releasing factors (CRF), which in turn stimulates the release of adrenocorticotropic hormone (ACTH) from the anterior pituitary gland (Wilmer et al., 2004). Once ACTH has been released it stimulates the adrenal cortex to release corticosterone (Peaker and Linzel, 1975; Bentley, 1976; Rankin and Davenport,

1981; Wilmer et al., 2004). Corticosterone mobilizes the energy stores of the animal to prepare for the flight/fight response.

Corticosterone is also very important in reptile and amphibian reproduction. While it has been positively correlated in amphibian reproduction, its correlation in reptiles is less clear (Zerani et al., 1991). Not all snakes react the same way physiologically to the presence of corticosterone; even within a species there are gender differences in how males and females react to the hormone. This lack of congruity in the function of corticosterone in reptiles makes it a subject of interest and wide study.

The presence of corticosterone does not always inhibit reproduction and/or the physiological changes that occur. The female red sided snake (*Thamnophis sirtalis parietalis*), emerges from the hibernacula with high levels of corticosterone, ready to mate (Whittier et al., 1987). In other snakes such as the western diamond-backed rattlesnake (*Crotalus atrox*), the females have higher levels of plasma corticosterone circulating towards the end of gestation (Taylor et al., 2004). This suggests that corticosterone may have a role in maintaining pregnancy in *C. atrox*.

Some anuran species, corticosterone is the foundation for the start and maintenance of reproduction, and the inevitable termination of the breeding season. Breeding males in the frogs *Hyla ebraccata*, *H. microcephala*, *Scinax boulengeri*, and *Physalaemus pustulosus* all have a positive relationship between advertisement vocalization, calling, and levels of corticosterone (Emerson and Hess, 2001). Male frogs are able to attract females to potential breeding ponds by calling, but these acts of advertisement are energetically costly for the male and alert predators to their location (Bucher et al., 1982). In order to mobilize energy reserves for the highly expensive cost

of calling, male frogs rely on corticosterone (Emerson and Hess, 2001). The presence of corticosterone contributes to the successful calling and mating. If the act of calling has depleted the energetic resources, the high concentrations of corticosterone will reach a threshold that suspends calling and ends the breeding season for that particular frog (Emerson and Hess, 2001).

The negative influence corticosterone has on reproduction is sometimes linked to the suppression of hormones associated with reproduction that influence behaviors that lead to mating (Moore and Jessop, 2003). The male red sided snake (*Thamnophis sirtalis parietalis*) and copperhead (*Agkistrodon contortrix*) exhibit high levels of corticosterone, which inhibit courtship behaviors crucial in their mating ritual (Friedman and Crews, 1985; Schuett et al., 1996; Moore et al., 2000; Schuett and Grober, 2000; Moore and Mason, 2001; Cease et al., 2007). In other snakes like *A. contortrix* the presence of corticosterone can affect the outcome of a fight. Males that lose fights were shown to have higher levels of circulating corticosterone in comparison to winners (Schuett et al., 1996; Schuett and Grober, 2000). A similar outcome is also seen in the male side-blotched lizard (*Uta stansburiana*), that competes with other lizards for territorial space (DeNardo and Sinervo, 1994).

Corticosterone not only has been studied in reproduction, but also in relation to stress. The acute or chronic consequences of stress can have bioenergetic implications that can affect the amount of energy the animal has to allot for reproduction, growth, and long and short term storage (DuRant et al., 2008). The energy constraints of corticosterone can affect the outcome of reproduction, but it may also pose long term effects on the progeny. Gravid garter snakes (*Thamnophis elegans*) were exogenously

treated with high doses of corticosterone and the outcome was an increase in embryonic mortality during gestation and after birth (Robert et al., 2008).

Other stressors that elicit the release of corticosterone are confinement or capturing and handling stress. The brown tree snake (*Boiga irregularis*) reacts to confinement stress by releasing corticosterone (Aldridge and Arackal, 2005; Mathies et al., 2001). The type of confinement stress, whether a trap or a bag made a considerable difference in the amount of corticosterone released (Mathies et al., 2001). Snakes that were confined to a bag were more stressed than those that were confined to a trap (Mathies et al., 2001). The release of corticosterone in response to capture and handling has also been shown to reduce testosterone levels in tuatara (*Sphenodon, punctatus*), wild alligators (*Alligator mississippiensis*), turtles (*Chrysemys picta*), and lizards (*Urosaurus ornatus*) (Licht et al., 1985; Lance and Elsey, 1986; Moore et al., 1991; Cree et al., 1990). Reptile corticosterone studies, to this point have not examined the effects of natural abiotic stressors on the hormonal stress pathway.

While *N. rhombifer* has a wide distribution across Texas and may be susceptible to temporary salt stresses, this species' salinity tolerance is unknown (Werler and Dixon, 2002). In addition, it is not known if exposure to salinity induces the activation of the stress hormone pathway in *N. rhombifer*. Salinity stress has been studied in ducks and it activates the stress hormone pathway (Peaker and Linzell, 1975). Unlike *N. rhombifer*, ducks have salt glands that aide in flushing out excess salts (Peaker and Linzell, 1975). Consequently, *N. rhombifer* is an ideal candidate for testing salt stress because of their large population distribution, potential natural exposure at the riverine/estuarine interface and the lack of information regarding salt stress in this species. The study proposes to (1)

evaluate the potential of salt stress to elicit the hormonal stress response in *N. rhombifer,* and (2) correlate corticosterone levels to the different salinity exposures in *N. rhombifer*. In order to determine if salt stress elicits the hormonal stress response in *N. rhombifer*, a repeated measures study was carried out testing salinity exposure at 0 ppt, 9 ppt, 18 ppt and 27 ppt. The data collected from this study will also likely be useful for explaining distributional limits for *N. rhombifer* along the Rio Grande and in other riverine systems.

# CHAPTER 2

# MATERIALS AND METHODS

## Animals and Maintenance

*Nerodia rhombifer* was selected as the species of study for this experiment because of its wide distribution and local abundance. The snakes that were tested were larger than 100 grams and appeared healthy. The four Hidalgo County, Texas sites chosen (Wallace Road, Edinburg Scenic Wetlands, Mercedes 1st waterfall, and Mercedes 3rd waterfall) each had an abundant population of *N. rhombifer*. I attempted to collect non-gravid females from the population and we limited our collection from each site to a maximum of ten individuals. The only site in which a collection was not made was in West Texas, Howard County; instead I purchased a male snake from this area. Howard County's water flows into the North Concho River and Morgan and Wild Horse creeks, all fresh water systems.

The Wallace Road site was a man-made lake surrounded by farm land with cattle grazing nearby, and from this site three males and three females were collected. The Edinburg Scenic Wetland site is a man-made lake in which its main water source comes from man-made canals and a water treatment plant, and from this site, two males we collected. The Mercedes 1st and 3rd waterfall sites are both surrounded by farm land and their main source of water comes from the Arroyo Colorado. Six males and two females were collected from the Mercedes 1st waterfall and 10 females were collected from the

Mercedes 3rd waterfall. The 27 animals were collected or acquired throughout the month of July from five different sites with a common freshwater environmental salinity. Water samples were taken from four of the five sites and a refractometer was used to measure salinity. The accuracy and sensitivity of the equipment was reduced in low concentrations of salinity, thus it approximately read close to 0 ppt for the four sites.

Shortly after capture, the animals were assigned a unique identification number, and gender was determined. The body weight, snout-vent length, tail length, width at the 13th lateral pre-cloacal band and head width measurements were taken using the squeeze box technique and recorded (Table 1) (Quinn and Jones, 1974). The following average measurements were taken of thirteen males (mass = 230.3 g ± 0.62 SE, snout-vent length = 59.6 cm ± 0.07 SE, tail length = 19.3 cm ± 0.02 SE, 13th band width = 3.00 cm ± 0.01 SE, and head width = 2.31 cm ± 0.01 SE) and of fifteen females (mass = 748.5 g ± 1.99 SE, snout-vent length = 81.7 cm ± 0.06 SE, tail length = 20.9 cm ± 0.02 SE, 13th band width = 4.45 cm ± 0.01 SE, and head width = 3.42 cm ± 0.01 SE). The females were significantly larger than males, which is consistent with the sexual size dimorphism characteristic of this species (Gibbons and Dorcas, 2004).

Twenty–eight plastic containers were used to house and test the animals in the experiment. A storage container was purchased with the following dimensions: 41.6 cm length, 29.2 cm width, and 14.9 cm height, and with a maximum volume of 13.6 liters. The dimensions of this container were suitable for this experiment because they were large and sturdy enough to hold a snake and four liters of water. The tops of these storage containers were altered for ventilation by cutting out a rectangular area on the top and covering the area with a metal hardware cloth that was held in place by eight

aluminum screws. The tops snapped easily on top of the container, but for extra security a string was wrapped tightly around the entire container.

In order to minimize fungal infections, also known as "blister disease", water was only offered *ad libitum* during feedings (Gibbons and Dorcas, 2004). The snakes were fed twice a week except during testing and a reasonable number of fish (either *Carassius auratus* (goldfish), or *Oreochromis aureus* (blue tilapia)) was offered. The number and size of the fish fed to any individual snake was deemed reasonable if it was equitable to the snake's body, thus larger snakes were offered larger fish or medium size fish, but in greater quantities. The *C. auratus* (goldfish) was purchased from Anderson Minnow Farm (www.andersonminnows.com) alive and frozen immediately. Since frozen fish have been known to cause thiamine deficiency in reptiles, the animal's diet was supplemented with live *O. aureus* (Beltz, 1989). The *O. aureus* for the feedings were collected from the Edinburg Scenic Wetlands using a net. Feeding frequencies were monitored and noted. Lastly, all of the animals were housed in a climate controlled room under laboratory temperatures (24-26˚C), and natural photoperiod regime.

## Experimental Protocol

The experimental protocol divided the experiment into four replicates with four groups, in which each *N. rhombifer* would be exposed to a specific salinity. The salt used for the different salinities was sodium chloride (NaCl) purchased online. Instant Ocean salt was not used for this experiment because it has other elements in it that might influence the results and other concurrent research is focusing on sodium relations. Exposure to the following salinities was tested: 0 ppt - similar to fresh water, 9 ppt -

equal to the isoosmotic level of blood, 18 - ppt twice the isoosmotic level of blood, and 27 ppt - three times the isoosmotic level of blood (Wilmer et al., 2004).

The salinity concentrations that were tested were not considered lethal to the snakes, since they were well below the salinity concentration, 35 ppt, of ocean water and had been previously studied in a pilot study of four *N. rhombifer* in which all the snakes survived. In an experiment performed on *Nerodia s. clarkii* and *Nerodia s. confluens*, conflicting results emerged with regard to the lethal salinity maximum of these two subspecies (Pettus, 1958). *Nerodia s. clarkii* was able to live in habitats with salinity as high as 73 ppt, but in its counterpart, *N. s. confluens*, the lethal salinity maximum was 30 ppt (Pettus, 1958). Therefore, due to the wide differences even within species, we assumed that anything under 30 ppt would not be lethal to *N. rhombifer*.

Stock solutions were made of the different salinities in order to facilitate access, standardize, and eliminate contamination. The following quantities were used to get the desired concentrations; for 0 ppt - 0g of salt and 18L of deionized water, for 9 ppt - 162g of salt and 18L of deionized water, for 18 ppt - 324g of salt and 18L of deionized water, and for 27 ppt - 486g of salt and 18L of deionized water. The water and salt were placed in a five gallon jug and mixed thoroughly. The accuracy of the salinity concentration made was checked using a refractometer.

The testing patterns were randomly chosen using a coin toss with the condition that the salinity for the replicate and group could not be the same (Table 2). Each replicate was tested for one week of constant exposure ensuring that the organism experienced the effects of the different salinity exposures. Thereafter, a two week resting period started for the snakes, in which they could recover and eat before starting the next

replicate. All of the snakes were eventually tested at all salinities in a repeated measures design except for the ones that perished. Since the test at 27 ppt salinity proved to be lethal in half of the snakes that were tested, I decided to terminate that portion of the experiment.

Before starting each replicate the animals were placed in fresh water for twenty four hours, thus ensuring that the snake was hydrated before starting the experiment. Blood was drawn from these animals to check for corticosterone, therefore 1ml syringes were purchased along with heparin. Heparin keeps the blood from coagulating in the syringe. Since heparin is sold in a concentrated powdered form, a solution of heparin was prepared; 0.015 g of heparin in 10 ml of deionized water was made to heparinize the needles. The needles used to draw blood were heparinized by drawing the prepared solution of heparin into the syringe and then injecting out the contents. The testing containers were labeled and four liters of the specific salinity exposure for each group respectively was added using the pre-made stock solutions. In addition, the temperature of the room was noted and closely monitored, since temperature may influence the amount of hormones circulating in the plasma (Krohmer et al., 1987).

An effort was taken to decreasing non-related experimental stress by minimizing handling time (Mathies et al., 2001). A stop watch was used to note the amount of time it took for the snake to be handled. The handling time started as soon as the snake was collected from its container, and ended when the snake had its sample taken and put back into its container. The snake was weighed and the weight recorded. A plastic tube was used to restrain the snakes while blood samples were taken.

In order to safely sample the blood, we collected less than 10% of the total blood volume at any given time, which equate to less than the 0.5mL of blood actually collected (considered to be a safe amount and total blood volume was estimated as 70mL $kg^{-1}$ (Dressauer, 1970). The total blood volume of the smallest snake was 6.5mL and largest was 107.1mL. The amount of blood drawn throughout the experiment was less than the 6% . Blood was drawn from under the fifteenth sub caudal scale or higher to reduce the damage to the hemipenal structures in males. Blood was collected from the ventral caudal vein, and its content was transferred to a microcentrifuge tube and placed on ice.

Once the blood was drawn the snake was immediately released from the plastic tubing and placed back into a plastic storage container. The same steps were taken when beginning and ending each replicate except the contents of the plastic storage container changed. If the replicate was beginning, then the container contained a specific salinity concentration and once the animal was placed in the container the level of the water was marked using a permanent marker. If the replicate was ending, then the container contained tap water aiding the animal in rehydration and recovery. The time along with the amount of blood collected before and after each replicate was recorded.

The microcentrifuge tube containing the sample was centrifuged at 1,000G for 15 minutes at 4ºC to separate the plasma from the cells. The plasma fraction was removed with a syringe and the amount collected was recorded. Both the cells and the plasma were stored separately in a -80 ºC freezer until the immunoassay was performed.

During the transition of the testing week observations were noted on the level of the water, condition of the animal, and whether there was any cloacal discharge. If the water level was lower than the mark initially made at the beginning of the experiment,

then using the osmometer the concentration of the salinity was checked and replaced. If the animal went through ecdysis, excreted or defecated in the container, then the container's content would be discarded and the snake would be placed in a fresh testing solution.

Finally, in order to analyze the plasma, a 480 well Corticosterone Enzyme Immuno Assay Kit was purchased from the Cayman Chemical Company (www.caymanchem.com). The kit uses a plate precoated with corticosterone antibody linked with an acetylcholinesterace tracer that in the presence of corticosterone lights up under a spectrophotometer (Catalog # 10005590, Cayman Chemical, Ann Arbor, MI). Separation of the corticosterone from the plasma binding proteins was accomplished using the buffers provided by the kit. An extraction test was performed previously for testosterone radioimmunoassay that determined there was a minimal amount of binding proteins in snake plasma to invalidate the kit (Zaidan et al., 2003). The cold extraction protocol was followed in this experiment and few modifications were made to the published Cayman Chemical Corticosterone EIA Kit protocol. The assay samples were randomized and were run in duplicates (N = 151 samples).

## Statistical Analysis

The data was $\log_{10}$ transformed prior to analyzing the corticosterone values. We conducted seven repeated measures ANOVA (SSAS Proc Mixed) to investigate the influence of corticosterone on the size of the snake, population, mass, handling time, number of blood draws, and treatments. A student's t-test was performed to identify gender differences with regard to mass and corticosterone. In addition, a student's t-test

was conducted for before and after corticosterone samples. The data collected from animals exposed to 27 ppt salinity were excluded because half of the animals tested did not survive, thus this outcome posed a logistical problem in analyzing the data in a repeated measures ANOVA.

**Table 1.** Standard data collected; gender was determined using a probe, mass measured using an electric balance, and snout-vent-length, tail length, 15th scale and head width was measured using a squeeze box technique (Quinn and Jones, 1974).

**Mercedes 1st Waterfall**

| ID | Gender | Mass (g) | SVL (cm) | Tail (cm) | 15th W (cm) | Head W (cm) |
|---|---|---|---|---|---|---|
| M3 | Male | 92.7 | 47.4 | 15.0 | 2.2 | 1.6 |
| M4 | Male | 292.2 | 65.6 | 18.0 | 3.6 | 2.4 |
| M5 | Female | 995.5 | 88.0 | 22.4 | 5.8 | 3.6 |
| M8 | Male | 211.7 | 60.6 | 17.8 | 3.0 | 2.0 |
| M9 | Male | 272.6 | 63.2 | 24.0 | 3.2 | 2.6 |
| M10 | Male | 182.3 | 58.6 | 20.6 | 2.1 | 2.8 |
| M11 | Male | 254.8 | 68.3 | 21.4 | 2.6 | 3.2 |
| M12 | Female | 1127.6 | 89.0 | 13.8 | 3.6 | 5.4 |

**Mercedes 3rd Waterfall**

| ID | Gender | Mass (g) | SVL (cm) | Tail (cm) | 15th W (cm) | Head W (cm) |
|---|---|---|---|---|---|---|
| O1 | Female | 1290.6 | 93.0 | 24.6 | 4.8 | 4.0 |
| O2 | Female | 381.9 | 74.2 | 14.8 | 3.2 | 3.2 |
| O3 | Female | 518.4 | 71.8 | 24.4 | 4.8 | 3.2 |
| O4 | Female | 196.5 | 59.8 | 15.0 | 2.6 | 2.2 |
| O5 | Female | 556.3 | 77.8 | 20.6 | 4.2 | 3.0 |
| O6 | Female | 1088.6 | 94.2 | 24.6 | 4.8 | 3.6 |
| O7 | Female | 572.2 | 82.4 | 20.4 | 4.3 | 3.5 |
| O8 | Female | 611.9 | 79.6 | 21.2 | 4.2 | 3.2 |
| O9 | Male | 141.2 | 48.0 | 13.2 | 2.2 | 2.2 |
| O10 | Female | 900.4 | 97.4 | 24.8 | 5.4 | 3.6 |

**Edinburg Scenic Wetlands**

| ID | Gender | Mass (g) | SVL (cm) | Tail (cm) | 15th W (cm) | Head W (cm) |
|---|---|---|---|---|---|---|
| D1 | Male | 406.9 | 71.2 | 23.8 | 3.4 | 2.2 |
| D2 | Male | 355.3 | 72.0 | 23.0 | 3.4 | 2.8 |

**Wallace Road**

| ID | Gender | Mass (g) | SVL (cm) | Tail (cm) | 15th W (cm) | Head W (cm) |
|---|---|---|---|---|---|---|
| W1 | Female | 726.4 | 88.2 | 23.2 | 4.4 | 2.8 |
| W2 | Male | 186.0 | 58.0 | 20.8 | 3.0 | 2.0 |
| W3 | Male | 198.2 | 54.8 | 18.8 | 3.2 | 2.0 |
| W4 | Female | 373.3 | 63.2 | 18.2 | 3.6 | 2.8 |
| W5 | Female | 1529.6 | 98.2 | 28.4 | 5.3 | 4.3 |
| W6 | Female | 358.7 | 68.5 | 17.6 | 3.9 | 2.9 |
| W7 | Male | 269.1 | 61.0 | 20.4 | 2.8 | 2.2 |

**West Texas**

| ID | Gender | Mass (g) | SVL (cm) | Tail (cm) | 15th W (cm) | Head W (cm) |
|---|---|---|---|---|---|---|
| 9L | Male | 131.1 | 46.0 | 14.0 | 4.0 | 2.0 |

**Table 2.** Experimental protocol used to determine the effects of salinity on the release of corticosterone.

| | **Rep 1** | | **Rep 2** | | **Rep 3** | | **Rep 4** |
|---|---|---|---|---|---|---|---|
| N=7 | 27 ppt Salinity | ***Rest for 2 weeks*** | 0 ppt Salinity | ***Rest for 2 weeks*** | 18 ppt Salinity | ***Rest for 2 weeks*** | 9 ppt Salinity |
| N=7 | 9 ppt Salinity | | 18 ppt Salinity | | 0 ppt Salinity | | ***** |
| N=6 | 0 ppt Salinity | | 18ppt Salinity | | 9 ppt Salinity | | ***** |
| N=7 | 18 ppt Salinity | | 9 ppt Salinity | | 0 ppt Salinity | | ***** |
| | ***1 week exposure*** | | ***1 week exposure*** | | ***1 week exposure*** | | ***1 week exposure*** |

# CHAPTER 3

# RESULTS

## Corticosterone EIA Kit

The standard curve for the Corticosterone EIA Kit used to analyze the samples was highly reliable ($R^2$=0.9775). Therefore, the concentration of corticosterone in each sample was determined by using the graph's absorbances and concentration of corticosterone [Fig. 2.]. Once the corticosterone concentrations of the sample were determined using the graph, the values were converted from pg/mL to ng/mL.

## Initial Analyses

A student's t-test was performed to identify any significant differences between the snakes gender and mass. The y-axis in the graph represents the mean mass in grams and in the x-axis the bars represent male and female snakes. Females were significantly larger than males ($t_{(26)} = 20.77$, $p < 0.001$) [Fig. 3]. The average mean mass for females was 748.5 g ± 1.99 SE and 230.3 g ± 0.62 SE for males.

A repeated measures ANOVA was performed to identify any significant differences in the size of the snake and the corticosterone concentrations. Preliminary analyses indicated that mass of the snake, did not significantly affect the corticosterone concentration ($p = 0.9511$). Therefore, the size factor was excluded in the subsequent analyses.

Survival

The probability of surviving was calculated for the 27 ppt treatment for each testing day, using a simple mathematical equation of dividing the number of snakes that survived over the total number of snakes for any particular day. The y-axis shows the probability of surviving by testing day. As the testing days progressed the likelihood of surviving 27 ppt was less than 50% [Fig. 4.], unlike the other treatments whose percent of survival for all testing days were 100%.

Population

A repeated measures ANOVA was performed to identify any significant differences in the delta corticosterone concentrations between the locations. The y-axis represents the delta corticosterone and the x-axis bars represent the locations; West Texas, Mercedes 1st Waterfall, Mercedes 3rd Waterfall, Edinburg Scenic Wetlands, and Wallace Road. There was no significant difference between the locations and the corticosterone response elicited after each treatment, ($F_{(4,18)} = 0.48$, $p = 0.748$) [Fig. 5.]. The mean corticosterone values for the sites were the following West Texas (-0.86 ng/mL ± 8.72 SE), Mercedes 1st Waterfall (4.12 ng/mL ± 2.46 SE), Mercedes 3rd Waterfall (3.58 ng/mL ± 2.15 SE), Edinburg Scenic Wetlands (0.01 ng/mL ± 3.86 SE), and Wallace Road (5.56 ng/Ml ± 2.17 SE).

Body/Weight Condition

A repeated measures ANOVA was performed to identify any significant differences in the delta mass between the treatments. The graph's y-axis contains the

delta mass values and the x-axis bars represent the treatments; 0 ppt, 9 ppt, and 18 ppt. There was no significant difference between the delta mass and treatments 0 ppt, 9 ppt, or 18 ppt ($F_{(1,39)} = 0.00$, $p = 0.951$) [Fig. 6]. The delta mean mass for the treatments were the following 0 ppt (1.87 g ± 2.31 SE), 9 ppt (-0.54 g ± 2.62 SE), and 18 ppt (6.11 g ± 2.55 SE).

Handling Time and Multiple Blood Drawings

A repeated measures ANOVA was performed to identify any significant differences in the time it took to draw blood from the animals between the treatments. The y-axis in the graph represents the time in minutes and the x-axis contains each treatment. There was no significant differences in the time it took to draw blood in the treatments 0 ppt, 9 ppt, or 18 ppt ($F_{(2,36)} = 0.20$, $p = 0.822$) [Fig. 7]. The mean time for all the treatments was 5.32 min ± 2.27 SE.

Another two repeated measures ANOVAs were performed to determine if repeated sampling affected corticosterone levels. The y-axis in the graph represents the concentration of corticosterone present and the x-axis contains the number of times blood had been drawn. Before salt exposure, baseline coticosterone levels were significantly higher in the first blood draw and subsequently lower in recurring ones ($F_{(2,39)} = 4.17$, $p = 0.023$) [Fig. 8.]. A Tukey's post hoc test was performed to differentiate between the groups and a significant difference was apparent in the corticosterone levels from the first and second time blood was drawn ($p = 0.0417$). In contrast, no significant effect of repeated sampling was detected after salt exposure ($F_{(2,39)} = 0.17$, $p = 0.843$) [Fig. 9.].

**Secondary Analysis**

A student's t-test was performed to identify any significant differences between the samples collected. The y-axis contains the corticosterone values and the x-axis bars represent the samples collected before and after each treatment. Corticosterone values were significantly higher after salinity exposure ($t_{(56)} = 9.29$, $p = 0.004$) [Fig. 10.]. The mean corticosterone value collected initially in each replicate was 5.07 ng/mL ± 0.94 SE and the final value was 12.76 ng/mL ± 2.37 SE.

Another student's t-test was performed to identify any significant differences between delta corticosterone and gender. The y-axis represents the delta corticosterone and the x-axis bars represent male and female snakes. There was no significant difference between males and females, ($t_{(18)} = 2.45$, $p = 0.135$) [Fig. 11.]. The delta corticosterone mean for males was 5.19 ng/mL ± 8.00 SE and -1.53 ng/mL ± 3.25 SE for females.

Finally, a repeated measures ANOVA was performed to identify any significant differences in salinity exposure and the change in corticosterone. Afterward, a Tukey's post hoc test was performed to differentiate between the groups. The y-axis contains the delta corticosterone values and the x-axis bars represent the different treatments 0 ppt, 9 ppt, and 18 ppt. There was a significant difference between salinities ($F_{(2,39)} = 3.55$, $p = 0.038$) [Fig. 12]. The Tukey's test further separated the salinities into different categories. A significant difference was noted between treatments 9 ppt and 18 ppt ($p = 0.034$), but not in treatments 0 ppt and 9 ppt ($p = 0.623$) and nor in treatments 0 ppt and 18 ppt ($p = 0.214$). The mean delta corticosterone for exposure to salinity 0 ppt was 1.87 ng/mL ± 2.31 SE, -0.54 ng/mL ± 3.31 SE for 9 ppt, and 6.11 ng/mL ± 2.55 SE for 18 ppt.

Figure 2. Standard curve acquired from the Corticosterone EIA kit. Approximately 98% of the variation in $B/B_0$ (absorbance ratio of a standard well to the maximum binding well) was explained by the corticosterone concentration, indicating a reliable standard curve within the observed limits.

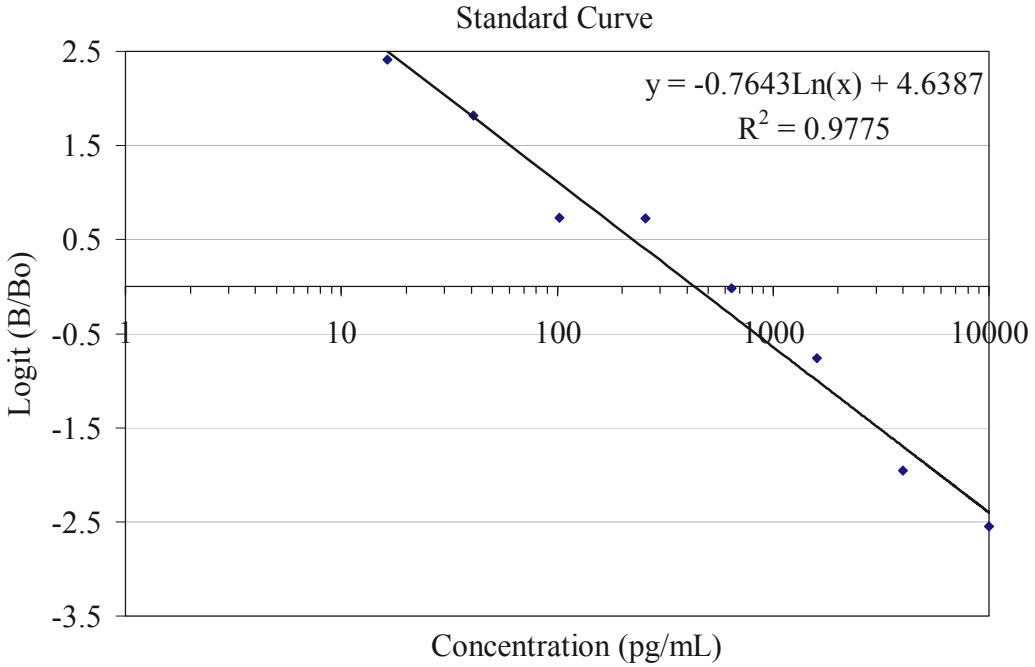


Figure 3. Comparisons of gender mass in the experimental animals. The error bars represent one standard deviation. Females are significantly larger than males, which is characteristic of *N. rhombifer*.

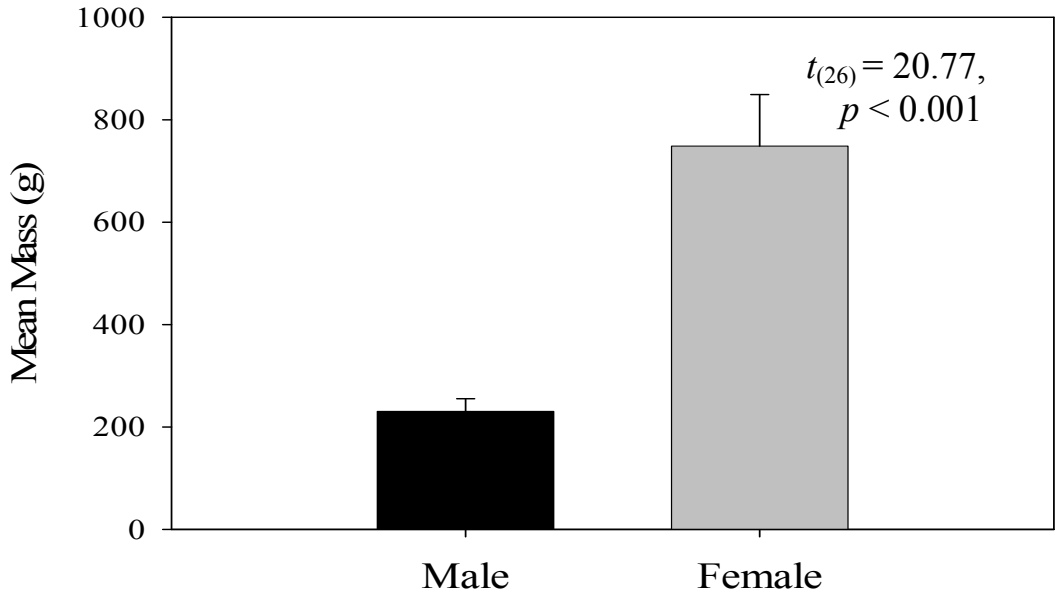


Figure 4. Comparison of days tested in 27 ppt to the probability of surviving exposure. As the days progressed, the likelihood of experimental animals surviving the 27 ppt treatment was less than 50%.

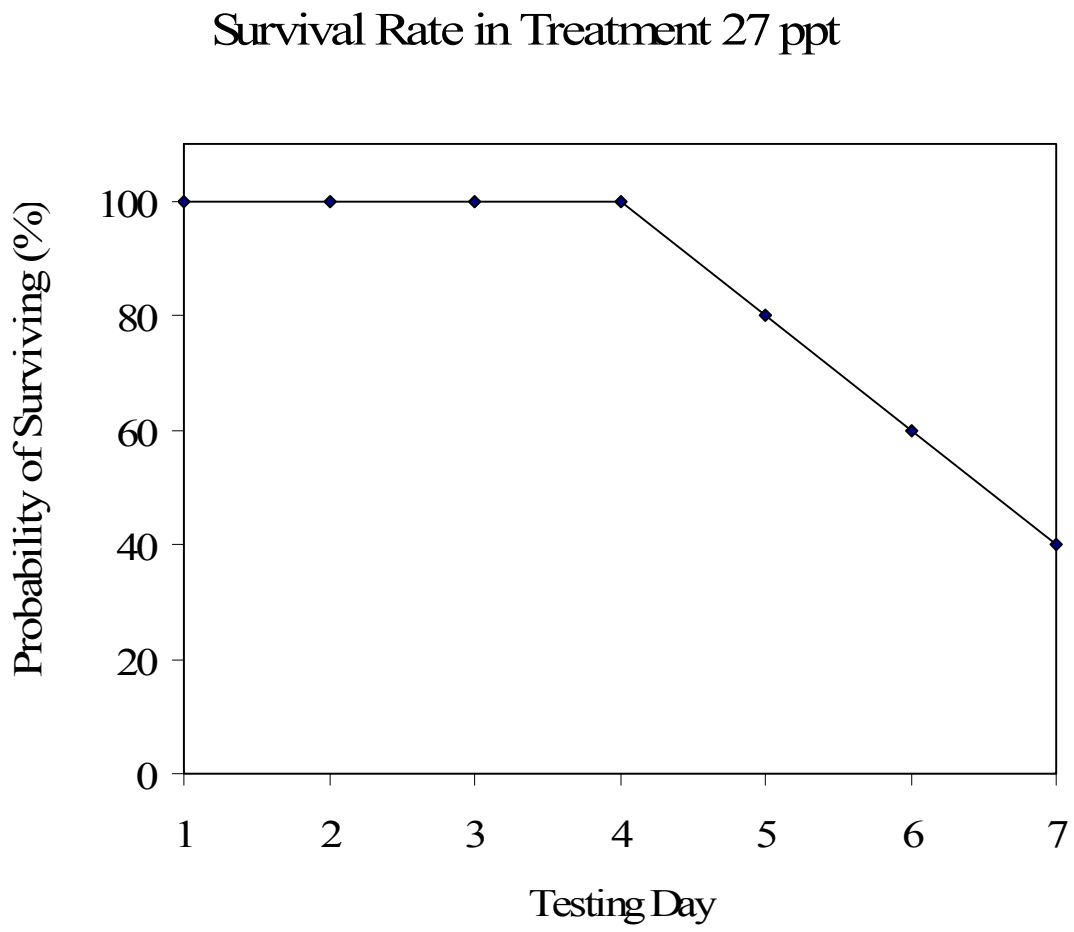


Figure 5. Comparison of the population-level of stress response to salinity exposure. The error bars represent one standard deviation. All five populations responded similarly to different salinity treatments.

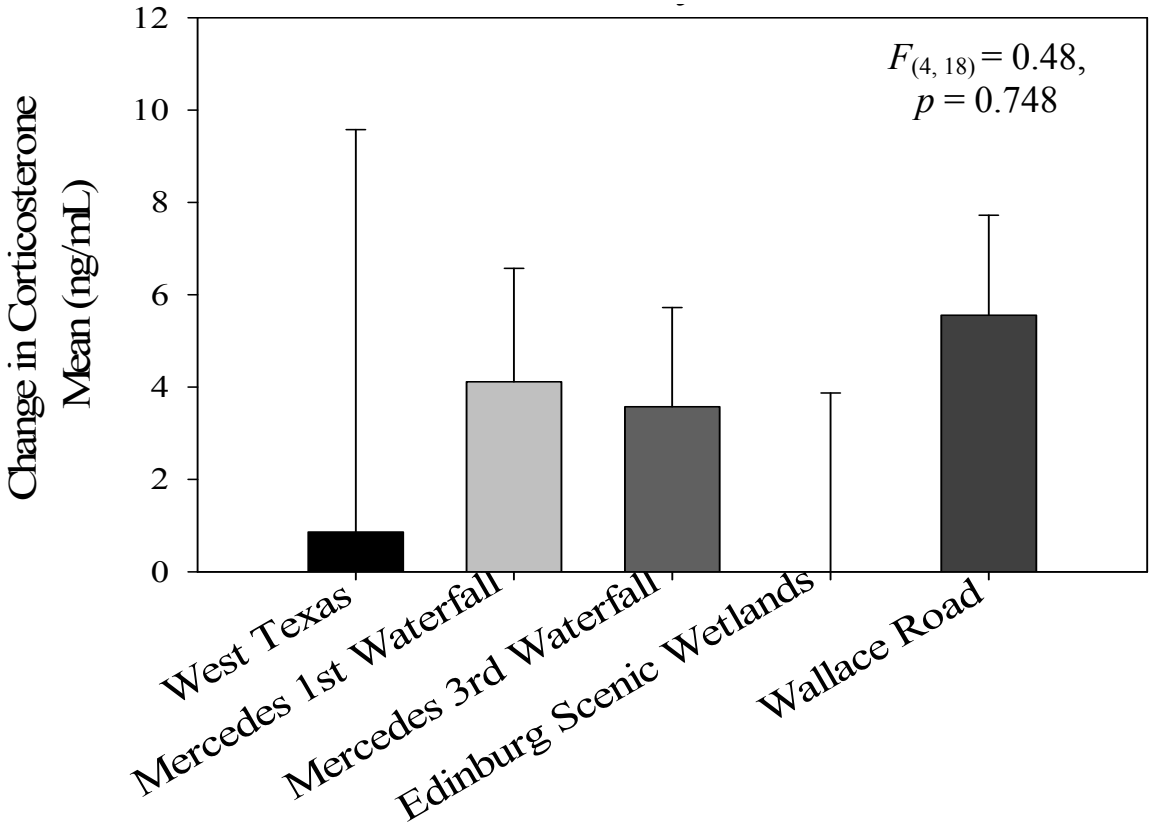


Figure 6. Comparison of changes in mass due to individual treatments. The error bars represent one standard deviation. Exposure to different salinity treatments did not affect the overall change in mass.

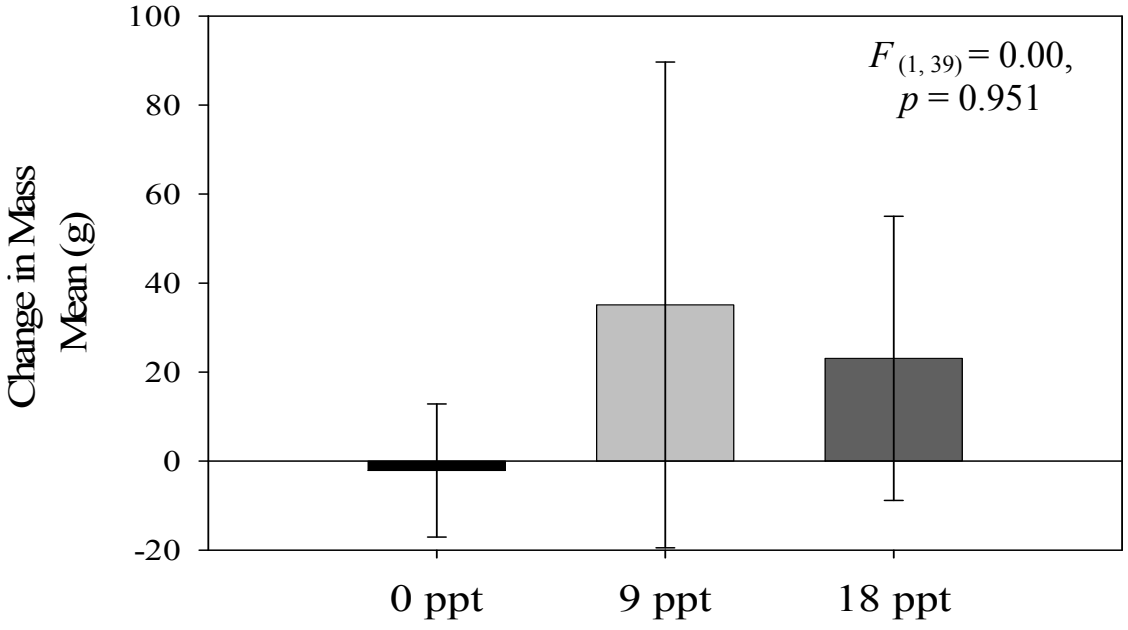


Figure 7. Comparisons of draw blood time across the different salinity exposures. The error bars represent one standard deviation. The time it took to draw blood in each individual animal in any particular treatment did not differ.

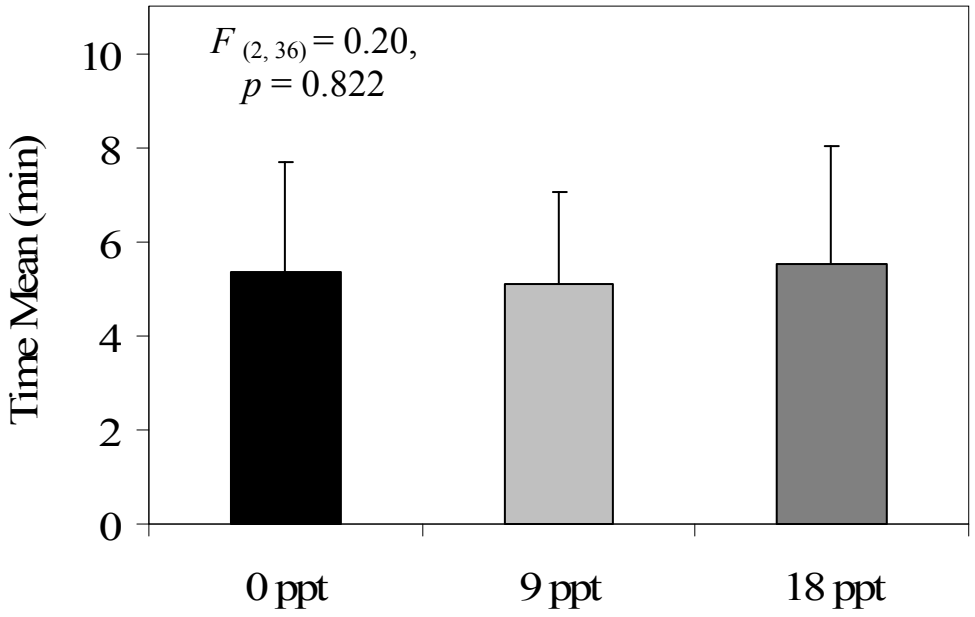


Figure 8. Comparisons of initial corticosterone across multiple blood drawings prior to salinity exposure. The error bars represent one standard deviation. The initial corticosterone concentration taken through out the treatments was significantly different.

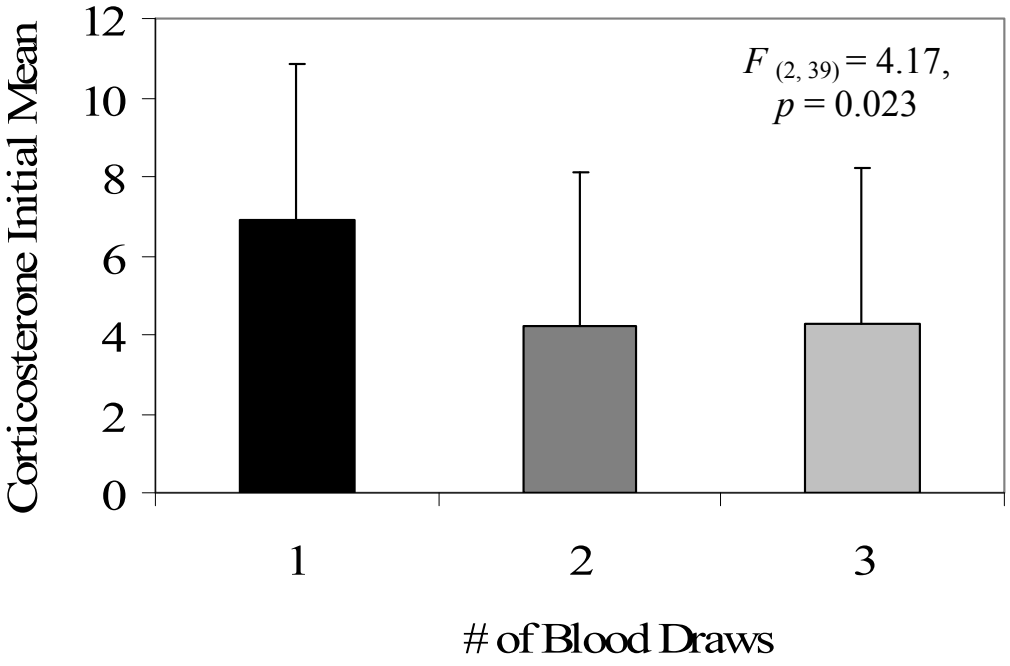


Figure 9. Comparisons of corticosterone levels after repeated blood drawings. The error bars represent one standard deviation. The corticosterone concentration after blood drawing was not significant.

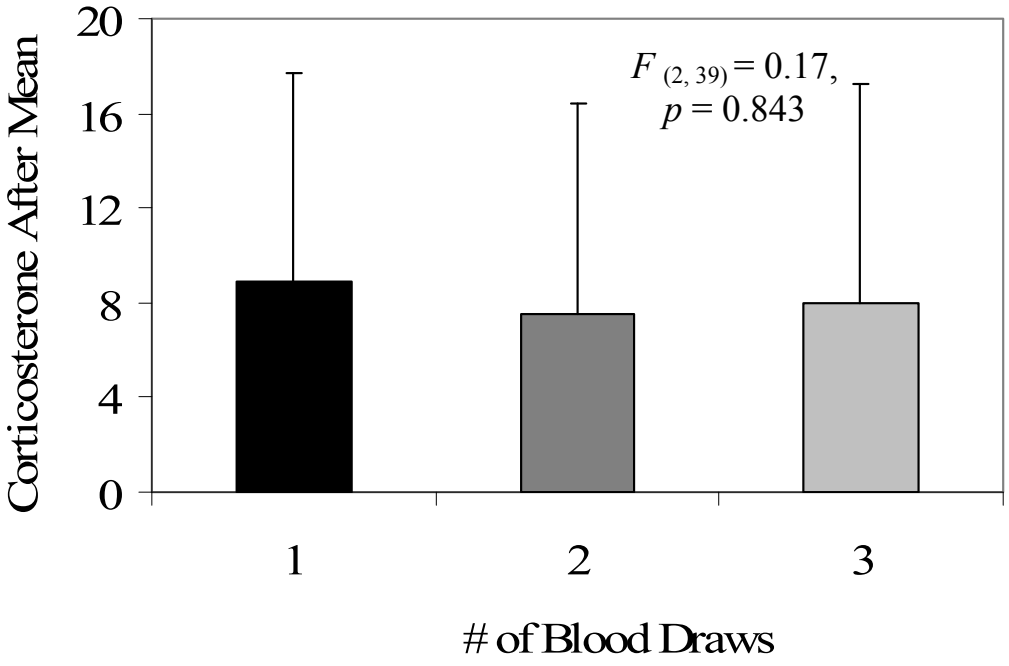


Figure 10. Comparison of corticosterone concentrations before and after any salinity exposure. The error bars represent one standard deviation. An increase in corticosterone was observed after exposure.

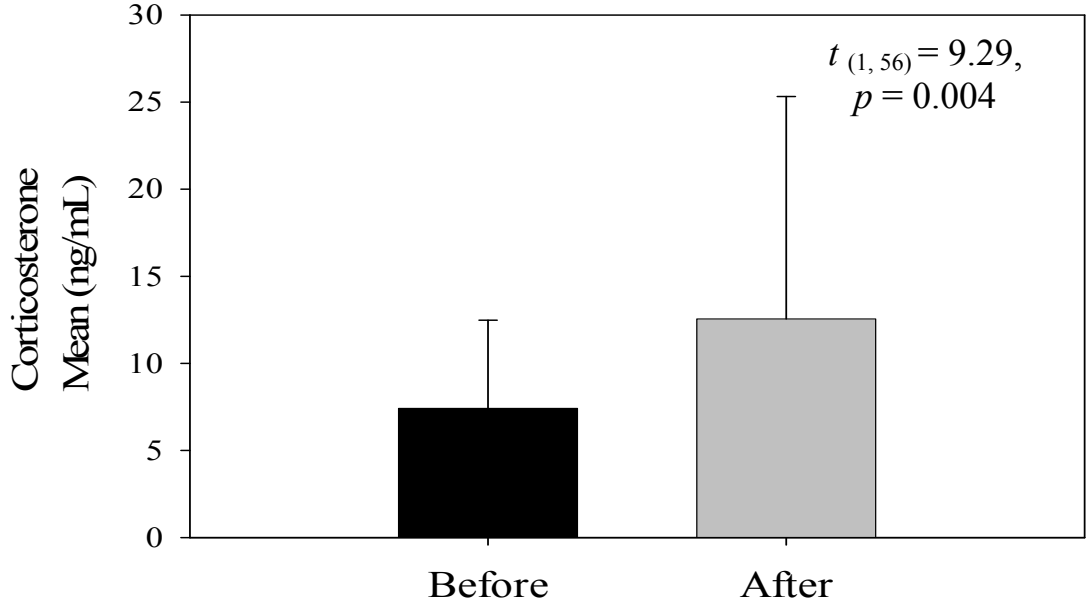


Figure 11. Comparison of gender effects on overall corticosterone concentrations. The error bars represent one standard deviation. The corticosterone response from both male and females were similar throughout the different salinity exposures.

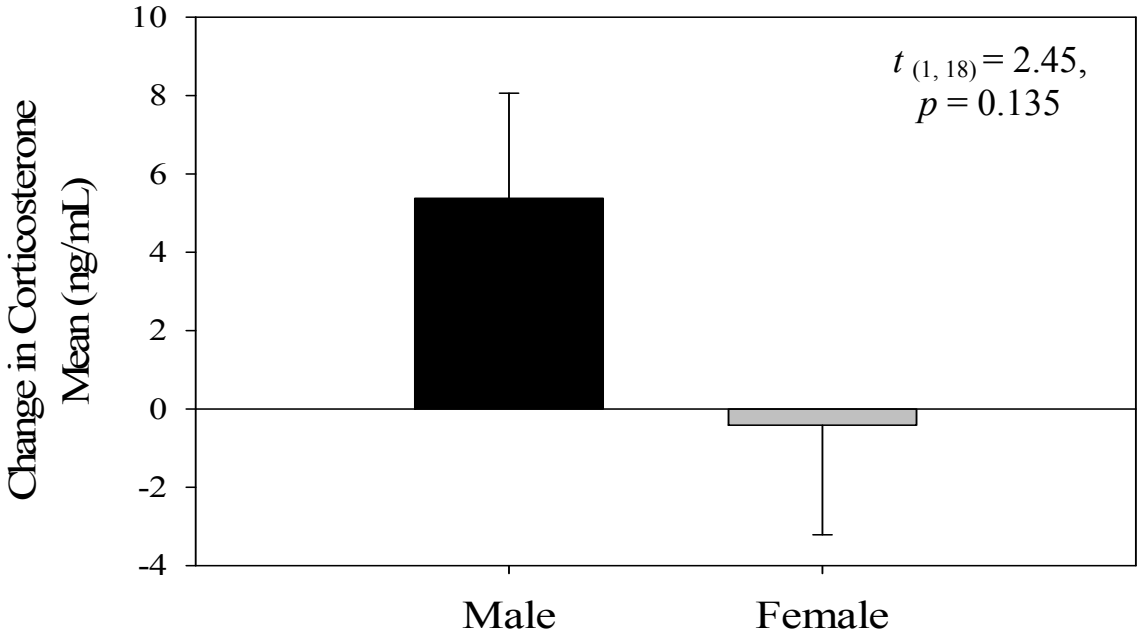
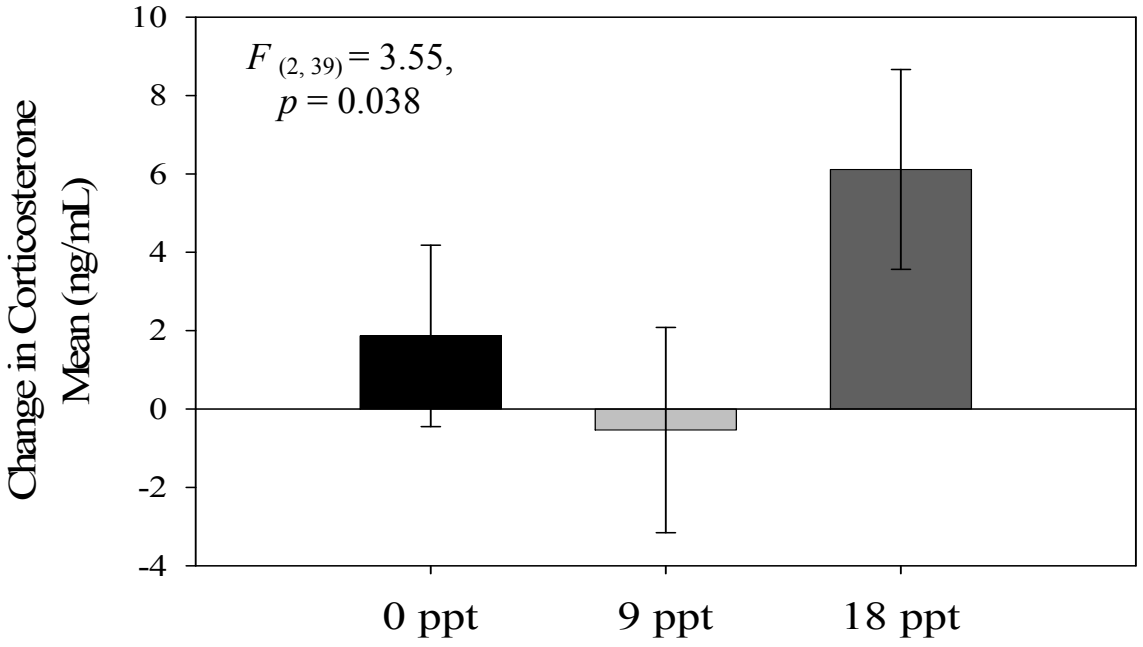


Figure 12. Comparison of corticosterone concentration changes due to the individual treatments. The error bars represent one standard deviation. The amount of corticosterone released was significantly different among the different salinity treatments.



# CHAPTER 4

# DISCUSSION

In an ideal setting, an animal is in an equilibrium state physiologically with its environment. There are always external forces that test the animal's ability to maintain homeostasis and these are considered stressors. In response to a stressor, an animal will mount a hormonal response in the form of corticosterone, but only if the stressor warrants a response. In this investigation, I tested if salinity stress would elicit the secretion of corticosterone.

The data strongly suggest that salt stress does elicit the hormonal stress response in *Nerodia rhombifer* and that the levels of corticosterone differ with salinity exposure. There was a significant difference in the animal's physiology once they had been exposed to salinity. I was able to analyze and quantify this by looking at changes in corticosterone.

The snakes exhibited sexual size dimorphism characteristic of a mature healthy adult population of *N. rhombifer* (Gibbons and Dorcas, 2004). *Nerodia rhombifer* also responded similarly as *N. confluens* to exposure to high salinity. *Nerodia confluens* was tested in 30 ppt salinity and they were able to survive a maximum period of four days (Pettus, 1958).

Using the General Adaptation Syndrome we can stipulate why death occurred in some of the snakes and not in others (Selye, 1936; 1946). The General Adaptation

Syndrome has three stages: an alarm stage where an animal exposed to a stressor produces an acute stress response; the resistance stage where the stressor becomes chronic but the adrenal glands are able to resist the stress physiologically by inhibiting growth and reproduction and; the last stage, exhaustion, where the stressor becomes chronic because the animal can no longer resist physiologically the stress and death occurs (Selye, 1936; 1946). The snakes that were able to survive testing at 27 ppt were able to recover because the stress had not become chronic, thus when the stressor was removed, in this case the salty water, they were able to survive. Other factors may have contributed to the demise or success of these snakes such as age, gender, sensitivity to the stress, and body condition (Selye, 1973; Wingfield and Romero, 2001; Moore and Jessop, 2003). The number of snakes studied at 27 ppt is not a large enough sample size to be able to clearly test for the previous factors or to draw distinct conclusions.

The data collected for the salinity treatments 0 ppt, 9 ppt, and 18 ppt was tested for population, body condition and gender effects. Even though these snakes were collected from different locations they all responded similarly when exposed to salinity. The response to salinity stress was apparently not affected by geographical location, environmental conditions, or genetic identity. These factors could have altered their adrenal response to stress (Dunlap and Wingfield, 1995; Moore et al., 2001; Moore and Jessop, 2003; Selye, 1973).

The mass of the snakes did not change with each individual salinity treatment. Therefore exposure to the different salinity treatments did not affect the overall change in mass. These results were unexpected since other studies testing salinity showed an overall mass change. The higher uptake of salty water should increase blood osmolality,

which in turn would activate thirst receptors and the animal would react by drinking more water (Bentley, 1976; Ranking and Davenport, 1981). In the salt marsh snake (*N. clarkii*) water balance was maintained by not drinking 30 ppt water, while in the broad banded water snake (*N. confluens*) balance was not maintained in the same situation because they drank the water (Pettus, 1958). A drastic change in mass was also noted in *N. confluens* shortly before death after being placed in 30 ppt (Pettus, 1958). The sea snake (*Hydrophis cyanocinctus*) experienced a slower rate of weight loss in freshwater, than in its normal estuarine environment (Duggan and Lofts, 1978). The results suggest three possibilities for our study: the animals recognized that the water was salty and ceased to drink, the animals did not drink enough salty water to activate thirst receptors, or the duration of the treatments was not enough to show mass differences.

The amount of time it takes to draw blood from an animal can produce stress that can alter the amount of corticosterone present. In this study, the time it took to draw blood across the treatments was done in relatively the same amount of time, on average, within five minutes. According to recent studies, corticosterone sample taken within 3 minutes confidently reflects the naturally unstressed levels of the animal (Romero and Reed, 2005). Although our average time is higher than the suggested time, it was consistent across all treatments.

The act of drawing blood can also stress the animal and alter the concentration of corticosterone present. Drawing blood did stress the animals, and was highest when blood was drawn for the first time prior to any salinity treatment, but afterward the animal's corticosterone concentration decreased. I analyzed the corticosterone concentration after the salinity treatment and there was no significant difference. This

suggests that the animal in the presence of two stressors: repeated blood drawings and salinity, salinity became the main stressor.

The corticosterone amount found in the initial sample of each treatment was considered the baseline for samples taken post treatment. The data collected from these samples showed that there is a significant difference after exposure to salinity. Salinity stress and the release of corticosterone had been studied extensively in ducks (Peaker and Linzel, 1975). In response to hypersaline injection the ducks released a comparable amount of corticosterone, which activated their nasal gland to secrete excess salt (Peaker and Linzel, 1975).

The results were crucial for this study, since not every stressor influences the release of corticosterone (Selye, 1936; 1946; 1973). Despite the fact that *N. rhombifer* elicited a stress response to salinities outside of their normal range, this does not mean that any salinity exposure outside of the animal's normal range would be stressful. The sea snakes (*Hydrophis cyanocinctus*) exhibited a decrease in corticosterone in the presence of fresh water (Duggan and Lofts, 1978). Likewise, in the sea turtle (*Lepidochelys kempi*) exposure to fresh water did not alter their corticosterone level (Ortiz et al., 2000). Maintaining the osmolality of blood can potentially be a source of stress, in the presence of salty water, for both freshwater and marine organisms.

The gender of an organism can also potentially affect the release of corticosterone (Selye, 1973; Moore and Jessop, 2003). In this study the snake's gender did not affect their response to the stress or the quantity of corticosterone released. Unlike *Sceloporus occidentalis*, *Chelonia mydas*, *Eretmochelys imbricata, Leidochelys olivacea*, *Thamnophis sirtalis parietalis*, and *Rana esculenta* in which the quantity of

corticosterone released was different in males and females (Whittier et al., 1987; Zerani, 1991; Dunlap and Wingfield, 1995; Valverde et al., 1999a, 1999b; Moore et al., 2000a; Jessop, 2001).

The amount of corticosterone released was significantly different among the different salinity treatments. Within the different treatments a significant difference was seen in 9 ppt and 18 ppt. The results suggest that *N. rhombifer* experienced an increase in circulating corticosterone in treatments 0 ppt and 18 ppt, but a decrease in corticosterone in treatment 9 ppt. If a stressor produces stress in an animal and stimulates the secretion of corticosterone, then *N. rhombifer* experienced stress in treatment 0 ppt and to a greater extent in treatment 18 ppt.

The decrease in cortcosterone in treatment 9 ppt is probably due to the lack of osmotic pressure experienced by the animal. One can assume that 9 ppt is less stressful than 0 ppt and 18 ppt. These results are congruent with data collected in other animals exposed to fresh water. The sea turtle (*L. kempi*) is adapted to salt water, but when exposed to fresh water failed to increase corticosterone levels (Ortiz et al., 2000). *Lepidochelys kempi* lacks the mechanisms to differentiate between fresh and saline water, therefore despite the animal's adaptations to minimize osmotic pressure in salt water, the fresh water treatment was less stressful (Ortiz et al., 2000). The sea snake (*H. cyanocinctus*) also experienced an overall lower osmotic pressure in fresh water and reduced its corticosterone levels (Duggan and Lofts, 1978). The favorable osmotic difference in environments coupled with the cessation of the functional use of the extra renal salt secreation helped *H. cyanocinctus* (Duggan and Lofts, 1978). The effects fresh

water had on both *L. kempi* and *H. cyanocinctus* can be an artifact of their evolutionary origin (Dunson and Mazzotti, 1989; Jackson et al., 1996).

The study of corticosterone is important because this hormone influences many factors: phenotype, genotype, body condition, reproductive fitness and other life history traits. The function of corticosterone in amphibians and reptiles is quite diverse. The link between stress and the release of corticosterone adds to the complexity of the issues. Stress in itself has been cited as antigonadal, hence detrimental for reproduction (Stephens, 1980). One could argue that reproduction alone is stressful and the act of mating inevitably releases corticosterone (Romero, 2002; Moore and Jessop, 2003; Graham et al., 2008). Despite citing corticosterone as deleterious for an organism's livelihood, corticosterone can be advantageous in other species by mobilizing energy reserves necessary for reproducing (Zerani et al., 1991). The influence of corticosterone is variable within the scope of time, season, individual, and context of the specific situations.

The largest potential source of error influencing the results in this study is the lack of life history information in these snakes. The influence of factors such as age, reproductive status, season, and to some extent genetic variability, are unknown. The age factor is important because it may have influenced the sensitivity to the stress (Selye, 1973). The reproductive status can modify the level of stress the animal experienced (Greenberg et al., 1984). Also the basal levels of corticosterone have been known to change from season to season (Moore and Jessop, 2003). The genetic variability can also potentially affect the modulation of the adrenal axis in response to stress (Moore and Jessop, 2003).

Since the snakes used in this experiment were wild caught, the effects of life history traits are unknown. The hormone corticosterone plays an important role in reproduction, and the snakes collected were all mature snakes. These snakes were collected during the mating season, thus some of the responses to salinity exposures may have been heightened. Although, we tried to collect non-gravid females, we later found out that half of the females were gravid. The gravid state may have also influenced their response to salt stress. All gravid snakes were able to complete full gestation and their litters were all apparently healthy. Ideally, all test individuals should have come from the same age that were not sexually mature, and were siblings. Initially an attempt was made to include snakes with the same life history traits, but their slow growth impeded the completion of this part of the experiment.

Further research should be conducted that controls for sexual status and the exposure time should be lengthened. The data collected would be able to properly depict the differences in prolonged exposures and could possibly clarify the results seen at 9 ppt. The duration of one week of salinity stress did not impede the gravid females from completing gestation, but the effects of salinity at the different gestations are unknown. Some of the males used in this experiment later had the opportunity to mate and produce viable offspring, therefore their mating capabilities were not hampered by the brief exposure to salinity stress. These need to be further explored in a controlled experiments and the results gathered could be incorporated into the general knowledge of the species.

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# BIOGRAPHICAL SKETCH

My name is Joana A. Cordoba and my permanent address 303 S. Bryan Blvd. Alton, TX. 78573. I was born in Miami, Florida and atended Florida International University (FIU). As an undergrad I worked with Dr. Eric Wagner at FIU as a research assistant in the Community Based Intervention Research Group. I received a degree from FIU a Bachelors of Science in Biology in the Spring of 2004. I participated in the HACU internship, where I did an internship with the Food and Drug Administration (FDA) in the Spring and another with the National Science Foundation (NSF) in Summer of 2004. My mentor, Dr. Kelman Wieder, at NSF asked me to work as a field research assistant in Canada collecting peet moss in the Spring of the same year. I took a leave of absence from my academic studies for a year, in which I worked at FIU as a teacher assistant and also a clinical assistant in the School of Health rehabilitation clinic. My experience with the internships motivated me to apply for grad school at the University of Texas – Pan American (UTPA). I started grad school in the Fall of 2005 and to support myself financially I taught 1402 Biology Labs and Comparative Vertebrate Anatomy Labs. In the Fall of 2008, while completing my thesis I took an internship with the United States Department of Agriculture (USDA) in the APHIS and later the ARS division. I worked at USDA/APHIS with Dr. John Goolsby on the Arundo Project for a year. Later I was invited to work with USDA/ARS with Josie Salinas on the The Rice Mite Project. Finally in the spring of 2009, I acquired from UTPA a Masters in Biology for my thesis; Stress responses to salinity in (*Nerodia rhombifer*).