EFFECTS OF EXPERIMENTALLY INCREASED TESTOSTERONE ON MOVEMENT AND THE EFFECTS OF SEX AND AGE ON RELATIVE CORTICAL VOLUMES IN THE NORTHERN PACIFIC RATTLESNAKE (*CROTALUS O. OREGANUS*)

A Thesis

Presented to

The Faculty of California Polytechnic State University

San Luis Obispo

In Partial Fulfillment

Of the Requirements for the Degree

Master of Science in Biological Sciences

By

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September, 2012

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ABSTRACT

Effects of Experimentally Increased Testosterone on Movement and the Effects of Sex and Age on Relative Cortical Volumes in the Northern Pacific Rattlesnake (*Crotalus o. oreganus*)

Julius A. Frazier

Hormones have been shown to play a crucial role in vertebrate reproductive behaviors. Androgens such as testosterone (T) stimulate male sexual behaviors such as mate-searching. Increases in movement and sex differences in navigational demands associated with increased movement are positively related to the volumes of cortical brain regions associated with navigational ability. In mammals and birds, the development and morphology of the hippocampal region, a structure within the forebrain, has been shown to play an important role in spatial learning, memory, and navigational ability. The medial cortex (MC), dorsal cortex (DC) and lateral cortex (LC) of non-avian reptiles are putative homologues to the avian hippocampus. This study sought to investigate the role of T in movement patterns displayed by free-ranging Northern Pacific Rattlesnakes (*Crotalus o. oreganus*) by utilizing a combined observational and experimental approach.

Twenty two adult male rattlesnakes were radio-tracked for four months. At the time of radio-transmitter implantation, twelve of the 22 snakes were randomly selected to receive testosterone implants (T-implanted group) and the remaining 10 snakes received blank implants (control group). We also quantified changes in concentrations of corticosterone (CORT) because CORT can suppress the gonadal axis, thereby functioning to inhibit gonadal hormone release and suppressing the stimulatory effects of T. In order to monitor plasma hormone concentrations over time, we collected blood samples at three times intervals throughout the study. Within the control group, T concentration was significantly positively correlated with several of our movement parameters. Conversely, within the T-implanted group, T concentration was not correlated with any of the movement parameters.

Additionally, we sought to investigate whether the difference in spatial ecology and associated navigational demands between the sexes is reflected in the relative volume of the MC, DC, and LC, and if sex differences in the relative volume of cortices are present from birth or develop later in life. For comparisons of adult DC, MC, and LC between the sexes, thirteen adult *C. o. oreganus* (6 females and 7 males) were collected in early May. In addition, two pregnant females were collected in early August of 2009 and held in the laboratory until parturition. A total of ten neonates (5 females and 5 males) were produced from the two litters. No sex differences in relative DC, MC, or LC were detected in adult or in neonatal rattlesnakes. Overall telencephalon volume was affected by sex in adult rattlesnakes (males larger) but not in neonates.

By utilizing a combined descriptive and experimental approach, we have shown that naturally occurring elevations in T are positively associated with certain measures of

movement and home range size. Failure of exogenous T in eliciting increases in movement suggests a steroid binding protein or T receptor saturation point. We show a non-significant trend in support of a complex relationship between the gonadal and adrenal systems, with experimentally increased T concentrations associated with a small increase in CORT. Finally, we suggest that changes in cortical volumes of *C. o. oreganus* are seasonal and occur over a relatively short time period. Results of this study highlight the need for further investigation into the timing and seasonality of volumetric changes in cortical volumes of reptiles, the mechanisms behind such changes, and further investigation into the neurological morphology of neonatal reptiles.

ACKNOWLEDGEMENTS

The completion of this thesis would not have been possible without the invaluable help of several people. First, many thanks to my advisor and committee chair, Dr. Emily Taylor, who provided amazing advice, guidance, support, and research assistance throughout the course of this project. Additionally, I want to thank Dr. Christy Strand for providing advice and much needed guidance during the laboratory portion of my study and Dr. Francis Villablanca for his comments during the preparation of this thesis. I would like to thank my lab mates Craig Lind, Mathew L. Holding, and Nicholas Pollock for their assistance in the field, advice, and support. I want to acknowledge the invaluable work of Ignacio Moore on the radioimmunoassays of our blood samples and to thank him for advice on the analysis of that data and interpretation of the results. Furthermore, I want to thank Amber M. Branske, Dustin Groh, and Jordan Ahle for assistance with data collection. Permission for collection of snakes was granted by the California Department of Fish and Game California (Scientific Collection Permit No. SC-8159). The care and use of the snakes was approved by the California Polytechnic State University IACUC (protocol No. 910). Lastly, this research was funded by the National Science Foundation, the American Society of Ichthyologists and Herpetologists, and the California Polytechnic State University Department of Biological Sciences.

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General Introduction

The types of movements animals make through the environment, the ultimate and proximate causes of such movements, and the effects these movements can have on the animals' morphology and physiology have been the focus of many scientific investigations. Animal movements are resource driven, and the need to reproduce has been implicated as a driving force behind many seasonal movements. Many studies have demonstrated that hormones play an important role in regulating vertebrate reproductive behaviors (Moore and Lindzey, 1992; Becker et al., 2002), and androgens such as testosterone (T) stimulate male sexual behaviors (Norris, 1997; Whittier and Tokarz, 1992). In general, an elevation in plasma T stimulates a variety of sexual behaviors and activities such as mate searching, territoriality, and aggression in males. In Chapter 1 of this thesis, I describe my use of observational and experimental techniques to investigate the role of T as a proximate cause of increased seasonal movements in male Northern Pacific Rattlesnakes *(Crotalus o. oreganus*). These seasonal movements place increased navigational demands on male Northern Pacific Rattlesnakes beyond those placed on females. Because females do not show seasonal increases in movement, male and female snakes of this species are under different navigational demands.

The proximate mechanisms by which the central nervous system stores and interprets information about the environment has emerged as a fruitful line of investigation in increasing our understanding of how animals are affected by and interact with their environment. Recently, it has been shown that parts of the vertebrate brain can response plastically to increased navigational demands (Sherry et al. 1993; Clayton 1995; Roth et al. 2006; Holding et al. 2012). Because male Northern Pacific Rattlesnakes

undergo seasonal increases in movement in search of receptive females, they must navigate through their environments more than females. In Chapter 2, I describe how I investigated whether this difference in movement patterns between the sexes is reflected in the volume of the navigational center of the brain and whether or not such differences are present at birth or develop later in life.

In summary, the major topics of this thesis center on animal movements. Chapter 1 describes the role testosterone plays as a proximate cause of movement where I use an observational approach by quantifying naturally occurring changes in testosterone concentrations and how this relates to seasonal changes in movement patterns, and an experimental approach to investigate the effects that experimentally increased testosterone can have on movement patterns. Additionally, Chapter 2 focuses on the effects that movement patterns can have on the morphology of the reptilian brain. Specifically, I use naturally occurring sex based differences in movement patterns to investigate the effects that differences in navigational demands can have on the relative volume of the navigational center of the brain. To do this, I measured various cortices known to aid in navigation within the telencephalon in adult snakes, when navigational demands are different between the sexes, and in neonates, before snakes experience navigational demands. Taken together, these chapters investigate the hormonal and neuroanatomical correlates of seasonal movements in the Northern Pacific Rattlesnake.

I. Effects of Experimentally Increased Testosterone on Movement in the Northern Pacific Rattlesnake (*Crotalus o. oreganus*)

Introduction

Many studies have demonstrated that hormones play an important role in regulating vertebrate reproductive behaviors (Moore and Lindzey, 1992; Becker et al., 2002). Androgens such as testosterone (T) stimulate male sexual behaviors (Norris, 1997; Whittier and Tokarz, 1992a). In general, an elevation in plasma T stimulates a variety of sexual behaviors and activities such as mate-searching, territoriality, and aggression in males. However, relationships between changes in steroid hormone concentrations and behaviors are complex in some reptiles (Sereau et al., 2010). The stimulatory effect of T on sexual behaviors has been validated for many species (Crews and Silver, 1985), but not all (King, 2002). Further, several experimental studies have shown that increased concentrations of gonadal steroid hormones are not required for sexual behaviors to occur during the mating season, but rather environmental cues such as photoperiod or ambient temperature may be more important in stimulating sexual behaviors (Camazine et al., 1980; Crews, 1994; Crews et al., 1984; Moore and Lindzey, 1992; Sereau et al., 2010).

Increases in activity, such as increased movement associated with mate-searching behaviors, increase energetic requirements placed on animals and often necessitate an increase in availability of energy reserves. Likewise, adverse weather conditions or changes in perceived predation risk often initiate mobilization of energy reserves thereby increasing energy availability. One mechanism to increase energy availability is via increases in concentrations of plasma glucocorticoids such as corticosterone (CORT)

(Marler and Moore, 1988; Wingfield et al., 1990; Folstad and Karter, 1992; Nolan et al., 1992; Wilson and Wingfield, 1994; Moore et al., 2000; Moore and Mason, 2001; Aubret et al., 2002; Cease et al., 2007). Increases in sexual behaviors not only increase energetic needs, but have also been associated with increased mortality (Bradley et al., 1980). Furthermore, elevated CORT can suppress the hypothalamo-pituitary-gonadal axis (Free and Tillson 1973; Moberg 1985; Sapolsky 1987), leading to deleterious effects on reproductive output.

Reptiles (particularly large-bodied viperid snakes) have emerged in recent years as very useful organisms for investigating the role of hormones in mediating reproductive cycles and behaviors in free-ranging animals (reviewed in Taylor and DeNardo, 2010). Due to their large body size and diverse mating systems, Crotaline snakes make an ideal model organism for studying the ecology and physiology of vertebrate reproduction (Beaupre and Duvall, 1998; Almeida-Santos et al., 2004; Schuett et al., 2006). Male Northern Pacific Rattlesnakes (*C. o. oreganus*) have larger home ranges than females (Putman et al., in review), partly because male snakes search for females and therefore exhibit large seasonal movements that increase home range size. Furthermore, because these large movements are temporally associated with annual increases in circulating plasma testosterone (T) concentrations (Lind et al., 2010), we have reason to hypothesize that these large movements and associated home range sizes are androgen-dependent. Male *C. o. oreganus* have two annual increases in circulating plasma T concentrations, one in the fall associated with spermatogenesis and mating, and another in the spring, associated with mating (Lind et al., 2010) but not spermatogenesis. Animals that show this bimodal type of mating system allow for investigations into the role of circulating

androgen concentrations in reproductive behaviors independently of other physiological mechanisms such as spermatogenesis. The present study addresses whether or not plasma T concentrations are associated with movement and home range size during the spring mating season in male *C. o. oreganus*. We combined a descriptive approach examining the relationship between naturally occurring T concentrations and spatial parameters with an experimental approach where T concentrations were manipulated. If T stimulates the increased movement and home range size observed in male snakes, then (1) there should be a positive correlation between T concentrations and spatial parameters such as movement distance and home range size in male snakes, and (2) males treated with T should move more often and/or longer distances and should have larger home ranges than control snakes.

Materials and Methods

Study site

We studied a population of *C. o. oreganus* on the northernmost portion of the Chimineas Ranch unit of the Carrizo Plain Ecological Reserve in the foothills of the Caliente Mountain range in San Luis Obispo County, California (35°N, 119°W). The study area was approximately 8 km^2 and is characterized by grazed plains and hills interspersed with oak and juniper woodland and prominent rock outcrops.

Animals and field monitoring

Twenty-two adult male *C. o. oreganus* were collected in April 2009 and transported to the laboratory at the California Polytechnic State University in San Luis Obispo where they were anesthetized with inhaled isoflurane gas. Mass (± 0.5 g) and snout-vent length (SVL, ± 0.5 cm) were measured while snakes were anesthetized using a digital scale and measuring tape. Snakes in this study averaged 97.0 ± 2.11 cm (standard error of mean, SEM) in snout-vent length (SVL) and 748.18 ± 44.23 g in mass (N=22). All snakes were reproductively mature, as *C. o. oreganus* reach sexual maturity at about 60 cm SVL (Diller and Wallace, 2002).

Each snake was injected with a passive integrative transponder (PIT) tag (AVID Identification Systems, Inc., Norco, California, USA) and given a unique three color rattle code for visual identification in the field. The rattle code was made by injecting acrylic paint into the three proximal rattle segments. Radio-transmitters (SI-2T 11-13 g transmitters, Holohil, Carp, Ontario, Canada) were implanted into the coelomic cavity (Reinert and Cundall, 1982). We radio-tracked the snakes 3-4 times per week from early May to early-September 2009 and used a global positioning system (GPS) unit (Garmin Etrex model # 010-00631-00) to record their locations with an accuracy of 6 m.

Hormone manipulation

At the time of radio-transmitter implantation, twelve of the 22 snakes were randomly selected to receive testosterone implants (T-implanted group) and the remaining 10 snakes received blank implants (control group). Each snake in the T-implanted group received two 1 cm long pieces of Silastic diffusion tubing (Dow

Corning, Clarksville, TN, U.S.A.: 1.47 mm inner diameter, 1.96 mm outer diameter) packed with crystalline testosterone (Sigma-Aldrich, St. Louis, MO, U.S.A.) with the ends of the tube sealed with silicone caulking (Momentive Performance Materials Inc. Huntersville, North Carolina). Snakes in the control group received two identical implants filled with silicone caulking instead of T. Implants were placed into the coelomic cavity alongside the radio-transmitters. After surgery, snakes were kept in the laboratory for 2-3 days for observation, and then released at the original sites of capture.

In order to monitor plasma hormone concentrations over time in the snakes, we collected blood samples at three times: early summer (May 29-June 11), late summer (July 10-13), and fall (August 26-September 3). Seasonal designations are biologically meaningful, based on changes in the movements and mating behavior of this population of snakes (Lind et al., 2010). Blood was collected from the caudal vein using a heparinized 1-cc syringe with a 25-gauge needle within five minutes of capture. Samples were placed in 1.5 ml centrifuge tubes and were centrifuged within 24 hours of collection. This time period is sufficient to ensure that steroid hormone concentrations are not altered (Taylor and Schuett, 2004). Plasma samples were stored at -20° C until radioimmunoassay. We were unable to collect a blood sample from one of the T-treated snakes because it was underground for an extended period, so the hormone data represent a sample size of 21 snakes.

Radioimmunoassay

Concentrations of T and CORT were measured by standard radioimmunoassay techniques following extraction and chromatographic separation as in Lind et al (2010).

For individual extraction efficiency determination, we equilibrated each sample over night with 2,000 cpm of tritiated steroid. Each sample was extracted with 5ml of distilled dichloromethane with the dichloromethane phase removed and dried in a warm water bath under a stream of nitrogen gas and resuspended in 10% ethyl acetate in isooctane. To remove neutral lipids and to isolate individual steroids, all samples were transferred to diatomaceous earth (Celite, Sigma) columns for chromatographic separation. Neutral lipids and other steroids were eluted with 2 ml of isooctane and discarded. Testosterone (T) and corticosterone (CORT) were eluted with 1.5 ml of 10%, 2 ml of 20%, and 2.5 ml of 50% ethyl acetate in isooctane, respectively, and saved. After this, samples were dried in a 40° C water bath under nitrogen gas, resuspended in 600 µl phosphate buffered saline, and maintained overnight at 4° C. Serial dilutions for the standard curves were performed in triplicate. All samples were then incubated overnight with 100µl of antiserum (T: T-3003, Wien Laboratories, Succasunna, NJ 07876; and B: Esoterix Endocrinology, Calabasas Hills, CA 91301) and 100µl of tritiated steroid. Unbound steroid was separated using dextran-coated charcoal and the bound steroid decanted into scintillation vials. Samples were counted on a liquid scintillation counter and final concentrations corrected for individual extraction efficiency.

Movement Parameters

Spatial data were analyzed using ArcView 3.3 software (ESRI Inc., Redlands, California, USA) with the Animal Movement Analysis extension (Hooge and Eichenlaub, 1997) and the Home Range extension (Rodgers and Carr, 1998). We calculated home range areas (ha) using the 100% and 95% minimum convex polygon (MCP) methods and

the 95% and 50% fixed kernel (FK) density estimates using the optimal smoothing factor with least squared cross-validation (LSCV) (Dugan et al., 2008). The 95% FK estimate is a measure of home range size comparable to the 100% MCP but is weighted by usage rate (density of observations within an area), and the 50% FK is a measure of core activity area.

Recent studies suggest that MCP is a more appropriate method for calculating home range sizes of snakes than the FK method because snakes often cluster around a single activity area, which tends to inflate the estimate produced by kernel analyses (Row and Blouin-Demers, 2006). Additionally, MCP only utilizes a fraction of collected data and does not account for animals revisiting an area several times, which snakes tend to do. However, FK estimates can be inconsistent among individuals due to the selection of an appropriate smoothing factor, but using LSCV is argued to provide the least biased results (Seaman et al., 1999). Taken together, MCPs are a more appropriate measure of home range area and FK is better for estimating areas of core activity. We report both FK and MCP estimates because each is an informative way of representing home range size, and so that these data can be compared to previous studies that may have only used one or the other.

We also recorded the distance between fixed locations for each snake and from this calculated mean distance traveled per day, frequency of movement, mean distance per movement, maximum distance moved, and total distance moved for each snake over the course of the study. Mean distance traveled per day was calculated by dividing the total distance moved during the study by the number of days the snakes were tracked. Frequency of movement was calculated by dividing the total number of movements

(greater than 6m, to account for GPS error and to eliminate movements of zero so as to not inflate this parameter) by the total number of fixed locations. Mean distance per movement was simply the mean of distances between fixed locations. The maximum distance moved was the single largest movement made by each snake. Total distance traveled was the sum of successive distances between fixed locations.

Data Analysis

We used repeated measures ANOVA in Statistica (StatSoft Inc. Tulsa, Oklahoma) with Tukey's post-hoc tests to detect the expected seasonal differences in T levels within and between treatment groups. We also used repeated measured ANOVA with Tukey's post-hoc tests to detect potential seasonal differences in CORT levels within and between treatment groups.

We used Pearson's correlation using Minitab 16.1.1 Statistical Software (Minitab Inc. State College, Pennsylvania) to test for effects of T concentration on 100% MCP, 95% MCP, 95% FK, and 50% FK, mean distance traveled per day, frequency of movement, mean distance per movement, maximum distance moved, and total distance moved. We also used Pearson's correlation to test for effects of CORT concentration on 100% MCP, 95% MCP, 95% FK, and 50% FK, mean distance traveled per day, frequency of movement, mean distance per movement, maximum distance moved, and total distance moved. Additionally, Pearson's correlation was used to test for effects of SVL on 100%MCP, 95% MCP, 95% FK, and 50% FK, mean distance traveled per day, frequency of movement, mean distance per movement, maximum distance moved, and total distance moved. In order to satisfy the assumptions of normality and

homoscedasticity, we square root transformed all of the home range estimates (100% MCP, 95% MCP, 95% FK, and 50% FK). Snake SVL was not correlated with any movement parameter and was therefore not included in movement analyses.

Results

Seasonal changes in plasma testosterone and corticosterone concentrations

The ANOVA model detected a significant effect of treatment ($F_{1, 24} = 6.96$, $p = 0.046$), time ($F_{2, 24} = 16.23$, $p < 0.001$) and a time by treatment interaction ($F_{2, 24} = 15.33$, $p < 0.001$) on T concentrations (Fig. 1). This indicates that T was different between the control and T-implanted groups, changed over time, and that the two groups changed differently over time. Specifically, T concentrations in the T-implanted group were significantly higher than controls during the early summer sampling period (May 29-June 11) ($p < 0.01$), but not in late summer and fall, likely due to natural increases in T in the control group during this time of year, associated with spermatogenesis (Lind et al., 2010).

The ANOVA model detected a significant effect of treatment on CORT concentrations ($F_{1, 29} = 11.80$, $p = 0.02$), but there was no effect of time ($F_{2, 24} = 0.38$, $p = 0.69$) nor was there a time by treatment interaction ($F_{2, 24} = 2.78$, $p = 0.12$) affecting CORT concentrations. Although post-hoc analysis did not detect any significant differences between treatment groups during the three seasons, the T-implanted group tended to have higher CORT than controls in early summer and late summer ($p = 0.056$ and 0.075 respectively; Table 1).

Because plasma T concentrations were significantly higher in the T-implanted group when sampled during the early summer period (May 29-June 11, Fig. 1), we are confident that the implantation technique was successful. Because T concentrations were only different in the early summer sampling period, statistical analyses related to movement patterns and T concentrations in T-implanted and control snakes are restricted to data collected during the early summer portion of the study (May 6-June 29). Importantly, the artificially increased concentrations of plasma T during this time period ranged within values recorded in later months (August-September) in control snakes and therefore allowed us to examine the results within a realistic (i.e., not pharmacological) hormonal context.

Effect of testosterone on movement and home range size

Within the control group, T concentration was significantly positively correlated with 100% MCP size ($r = 0.72$, $p = 0.02$, Fig. 2), mean distance moved per movement ($r = 0.67$, $p = 0.04$, Fig. 3), maximum distance moved ($r = 0.3$, $p = 0.0001$, Fig. 4), and total distance moved ($r = 0.65$, $p = 0.04$, Fig. 5), but not with the other variables: 95% MCP ($r = 0.57$, $p = 0.09$), 95% FK ($r = 0.47$, $p = 0.18$), 50% FK ($r = 0.33$, $p = 0.35$), mean distance traveled per day ($r = -0.07$, $p = 0.86$), and frequency of movement ($r = 0.30$, $p = 0.40$). In the T-implanted group, T concentration was not correlated with any of the home range or movement parameters: 100% MCP size ($r = -0.12$, $p = 0.60$, Fig. 2), 95% MCP ($r = 0.03$, $p = 0.91$), 95% FK ($r = -0.04$, $p = 0.88$), 50% FK ($r = -0.04$, $p = 0.86$), mean distance traveled per day ($r = 0.16$=, $p = 0.64$), frequency of movement ($r = -0.27$, $p = 0.42$), the mean distance moved per movement ($r = 0.23$, $p = 0.31$, Fig. 3), maximum distance moved ($r = -0.30$, $p = 0.34$, Fig. 4), and total distance moved ($r = -0.18$, $p = 0.62$,

Fig. 5). Mean hormone concentrations and spatial parameter values for T-implanted and control snakes during the early summer sampling period are shown in Table 1.

Effect of corticosterone on movement and home range size

In the control group, CORT concentration was not correlated with any of the home range or movement parameters: 100% MCP size ($r = -0.35$, $p = 0.33$), 95% MCP ($r = 0.03$, $p = 0.41$), 95% FK ($r = -0.12$, $p = 0.63$), 50% FK ($r = 0.06$, $p = 0.86$), mean distance traveled per day ($r = -0.05$, $p = 0.89$), frequency of movement ($r = 0.34$, $p = 0.30$), the mean distance moved per movement ($r = -0.37$, $p = 0.30$), maximum distance moved ($r = -0.56$, $p = 0.10$), and total distance moved ($r = -0.38$, $p = 0.23$). Likewise, in the T- implanted group, CORT concentration was not correlated with any of the home range or movement parameters: 100% MCP size ($r = -0.14$, $p = 0.68$), 95% MCP ($r = 0.23$, $p = 0.50$), 95% FK ($r = -0.06$, $p = 0.87$), 50% FK ($r = -0.03$, $p = 0.94$), mean distance traveled per day ($r = -0.57$, $p = 0.07$), frequency of movement ($r = -0.23$, $p = 0.43$), the mean distance moved per movement ($r = -0.12$, $p = 0.75$), maximum distance moved ($r = 0.001$, $p = 0.99$), and total distance moved ($r = -0.12$, $p = 0.73$).

Discussion

Free-ranging male *C. o. oreganus* had relatively low T concentrations during early summer (May 29 – June 11), with levels increasing during late summer (July 10 – 13) and into fall (August 26 – September 3). In this population, snakes experience two annual peaks in T (Lind et al., 2010): one in the spring (before the present study began) and one in the late summer and early fall (as observed in the present study). In control

snakes, we observed the predicted relationship between T concentrations and several of the spatial parameters measured: higher T was associated with larger 100% MCP size, longer average distances per movement, larger movements in the distribution of distances moved, maximum distance moved, and a greater total distance moved throughout the study period. Importantly, it should be noted that movement parameters based on calculated distances between fixes are not independent of each other. However, these different parameters allowed use to investigate the way snakes moved and utilized their space resources, and not just the distances they covered and areas they occupied. We were likely able to detect differences in these parameters and not the others because they encapsulate the rare long distance forays made by individual snakes with the highest T, and are measures of actual movement rather than movement parameters that are corrected for the number of days (mean distance traveled per day) or number of fixed locations (frequency of movement). In contrast, the 95% MCP and the FK methods would explicitly exclude these data points. Because individual snakes may have only displayed one or two long movements over the course of the study, these forays were likely masked in the mean distance per day parameter. Therefore, we suggest that the increases in movement associated with higher T are likely due to increased mate-searching effort manifested in a small number of forays.

Association between elevated T and male mating behavior has been reported for several other species of pit vipers (copperheads, *Agkistrodon contortrix*, Schuett, 1997; Mohave rattlesnakes, *C. scutulatus*, Schuett et al., 2002; Western Diamond-backed rattlesnakes, *C. atrox*, Taylor et al., 2004), as well as other species of snakes (Weil and Aldridge, 1981; Aldridge et al., 1990; Ernst and Ernst, 2003), fishes (Oliveira et al.,

2002), lizards (DeNardo and Sinervo, 1994; Moore and Marler, 1987, Moore, 1987a), birds (Chandler et al., 1994; Moss et al., 1994; Hau et al., 2000), and mammals (Cavigelli and Pereira, 2000; Gorden et al., 1976). Nelson (1994) reviews the extensive evidence for the role of androgens, such as T, in the expression of reproductive behaviors. Here we report another example of temporal association between elevated T and increased movement. In this case, male *C. o. oreganus* demonstrate long distance forays associated with elevated levels of T during a time of year in which mating occurs (Lind et al., 2010), suggesting that the snakes were searching for receptive females. This provides partial support for the hypothesis that T stimulates increased movement and home range size in rattlesnakes.

Although natural T concentrations in the control group were positively correlated with several of the spatial parameters we measured, we did not observe increased movements or home range sizes in snakes with experimentally elevated plasma T concentrations. This result does not support the hypothesis that T stimulates increased movements. It is possible that once a certain high concentration of T is reached, further increases in T fail to elicit concomitant increases in movement or home range size. For example, the T-treated snakes may have experienced a saturation point in steroid binding proteins or androgen receptors, in which further increases in plasma T beyond approximately 70 ng/ml no longer elicited a behavioral response in these snakes. All of the T-implanted snakes had T concentrations above 70 ng/ml (70.67 to 138.21 ng/ml), and exhibited no relationship between T concentrations and spatial parameters, whereas during early summer sampling the control snakes all had T levels below 70 ng/ml (2.74 to 67.88 ng/ml) and showed a positive relationship between T concentration and several

movement parameters. Swett and Breuner (2008) reported that approximately 96% of T was bound to corticosterone-binding globulin (CBG) in white-throated sparrows, suggesting that CBG levels may influence the availability of T to tissues and possibly define a threshold in the effects of T on behavior. Furthermore, steroid binding globulin levels and actions can change seasonally, in response to environmental changes (Breuner and Orchinik, 2002), day length, and T concentrations (Deviche et al., 2001). Because there have been very few studies investigating steroid hormone receptors in snakes (Kleis-San Francisco and Callard, 1986; Riley and Callard, 1988), and only a single study on snake steroid hormone binding proteins (Riley et al., 1988), further investigation of these topics is clearly needed.

Another reason experimentally increased T may have failed to elicit increased movement could be the extremely dry conditions in 2009. According to the California Department of Water Resources' weather station at La Panza (Latitude: 35.3806 Longitude: -120.1881 Elevation: 1650 feet), the closest weather station to our field site, this area received 12.47 inches of rainfall in 2008, only 9.83 inches in 2009, and 24.26 inches in 2010. The dry conditions of 2009 are reflected in the total distance moved by male *C. o. oreganus*. That is, the average total distance moved in 2008 and 2010 was 2046.3 and 1965.4 meters respectively, whereas in 2009 the average total distance moved was only 793.2 meters. Because snake movements appear to have been greatly diminished in spring 2009, perhaps due to the prolonged drought, it is possible that male snakes would have been more responsive to increases in T under wetter conditions but instead remained sedentary in underground refugia due to severe weather conditions, presumably in an attempt to mitigate desiccation.

Quantification of changes in concentrations of glucocorticoids such as CORT is essential in studies investigating seasonal fluctuations in T, experimentally manipulated T, and the possible effects of T on behaviors like movement patterns for two main reasons: (1) changes in behavior associated with elevated T, such as increases in movement patterns, can increase energetic demands, thereby eliciting an increase in CORT concentrations, and (2) CORT can suppress the hypothalamo-pituitary-gonadal axis (Free and Tillson 1973; Moberg 1985; Sapolsky 1987), thereby functioning to inhibit gonadal hormone release (Sapolsky et al. 2000) and suppressing the stimulatory effects of T. Our results mirror those of Taylor et al. (2004) in that CORT levels did not change seasonally, indicating that temporally constrained activities such as mate-searching did not elicit a change in circulating CORT. The T-treated snakes in this study showed a trend (although not statistically significant) of higher circulating levels of CORT than control snakes in the early and late summer sampling times. This result coincides with the findings of Ketterson et al. (1991) and Schoech et al. (1999) who reported that exogenous T was associated with elevated glucocorticoid concentrations in birds. Additionally, experimental elevation of T may have increased the snakes' metabolic rates, an effect observed in several species of birds (Zysling et al., 2006; Tobler et al., 2007, but see Buttemer and Astheimer, 2000). If elevation of T increased the snakes' metabolic rates, we may have elicited an energy mobilization response via a small increase in circulating CORT. Although male *C. o. oreganus* show increased activity during the breeding season via increased movement while searching for females, we did not detect a statistically significant increase in CORT in the present study. This finding suggests that energetic requirements of male *C. o. oreganus* are not substantially increased during the breeding

season. Beaupre and Duvall (1998) suggested that the low metabolic rates and energy requirements of rattlesnakes, when compared to other reptiles of similar size, may allow them to meet increased energetic demands without a concomitant increase in energy mobilization via increases in CORT concentrations.

In summary, by utilizing a combined descriptive and experimental approach, we have shown that naturally occurring elevations in T are positively associated with certain measures of movement and home range size, lending support to the hypothesis that T stimulates increased movement and home range size in rattlesnakes. Additionally, we show a non-significant trend in support of a complex relationship between the gonadal and adrenal systems as suggested by Romero (2002), with experimentally increased T concentrations associated with a small increase in CORT. We suggest that the failure of exogenous T to elicit increases in movement and home range size may be due to a steroid binding protein or T receptor saturation point, highlighting the need for more studies investigating possible thresholds, as this line of investigation is likely to be fruitful in increasing our understanding of the relationships between hormone levels and behaviors in free-ranging animals.

II. Effects of Sex and Age on Relative Medial, Dorsal and Lateral Cortex Volume in the Northern Pacific Rattlesnake (*Crotalus o. oreganus*)

Introduction

In mammals and birds, the development and morphology of the hippocampal region, a structure within the forebrain, has been shown to play an important role in spatial learning, memory, and navigational ability (O'Keefe and Nadel, 1978; Morris, 1982; Sutherland et al., 1983; Sherry and Duff, 1996; Burgess et al., 1999; Hollup et al., 2001). Additionally, the relative size or volume of the hippocampal region has been positively correlated with ability to perform spatial tasks (Crusio and Schwegler, 1987; Crusio et al., 1987; Biegler et al., 2001). Recent studies have elucidated potential links between hippocampal volume and aspects of spatial ecology in many taxa, including voles (Jacobs et al., 1990), lizards (Day et al., 1999a), birds (Shettleworth, 2003), and most recently snakes (Roth et al. 2006; Holding et al. 2012).

Individuals that occupy large home ranges, such as polygynous voles compared to monogamous voles, have enhanced spatial abilities (Gaulin and Fitzgerald, 1986) and larger hippocampal regions (Jacobs et al., 1990). Investigations from laboratory studies on mammals have been instrumental in understanding the role of the hippocampus in spatial learning, and much of what we know about the relationship between navigational ability and hippocampal size in free-ranging animals comes from a variety of studies on food-storing birds. Food-storing birds have larger hippocampi than non-food-storing birds, as the former are dependent on their ability to relocate storage sites and therefore experience greater navigational and memory demands (Krebs et al., 1989; Clayton, 1995;

Lucas et al., 2004; but see Volman et al., 1997; Brodin and Lundborg, 2003). Likewise, food-storing species of birds within the family Corvidae that store more food have larger relative hippocampi than those that store less food (Healy and Krebs, 1992, 1996; Hampton et al., 1995; Basil et al., 1996). Birds that parasitize the nests of other birds have larger hippocampi than birds that do not (Sherry et al., 1993; Reboreda et al., 1996; Clayton et al., 1997). In humans, taxi cab drivers have increased navigational demands associated with spatial learning and memory, routinely perform extensive spatial tasks, and also possess larger relative posterior hippocampi than controls (Maguire et al., 2000). Volume of the posterior hippocampus positively correlates with the amount of time spent as a taxi cab driver. Likewise, taxi cab drivers have larger posterior hippocampi than bus drivers, presumably due to increased navigational demands, as bus drivers follow the same route over and over whereas taxi cab drivers do not (Maguire et al. 2006).

The medial cortex (MC), dorsal cortex (DC) and lateral cortex (LC) of non-avian reptiles are putative homologues to the avian hippocampus (Butler and Hodos, 1996). However, few studies to date have investigated the structure, function, or ecological relevance of these cortices. Disruptions in cognitive spatial tasks have been achieved by lesioning the MC and DC of turtles (Grisham and Powers 1989; Rodriguez et al. 2002a; Lopez et al. 2003b) and the MC of lizards (Day et al. 2001; Lopez-Garcia et al. 2002). Two studies to date have investigated the relationship between navigational demands and cortex volume in snakes. Roth et al. (2006) showed that male cottonmouth snakes have larger MC, but not DC, than females. This larger MC volume is again associated with increased navigational demands, as male cottonmouths have larger home ranges than females. Holding et al. (2012) experimentally increased navigational demands via short

distance translocation and reported a concurrent increase in MC volume in Northern Pacific Rattlesnakes (*Crotalus o. oreganus*). Descriptive and experimental studies like these are instrumental in broadening our understanding of the relationships between cortical size and ecological demands. Furthermore, because few descriptive or experimental studies of this type have been performed on non-avian reptiles, and because the few performed have focused on adults, there is clearly a need for further investigation into a greater diversity of taxa and age classes with regard to relationships between spatial ecology and neural morphology.

Many viperid snakes make long and/or frequent movements associated with mating, making them a good model organism for studying the neural responses to ecological demands. Male *C. o. oreganus* occupy much larger home ranges than females, and this sex difference in their spatial ecology is more pronounced during the spring mating season than in other times of year (Putman et al. in review). The aim of this study was to further elucidate whether the difference in spatial ecology between the sexes is reflected in the relative volume of the MC, DC, and LC. If so, then are sex differences in the relative volume of cortices present from birth or do they develop later in life? We hypothesized that cortical volumes increase in response to increased spatial demands. If this is true, then adult males should have larger cortical volumes than adult females, and there should be no sex difference in cortical volumes of neonates because they have not yet experienced navigational demands.

Materials and Methods

Animal collection and handling procedures

For comparisons of adult DC, MC, and LC between the sexes, thirteen adult (SVL > 60 cm) (Diller and Wallace, 2002) *C. o. oreganus* (6 females and 7 males) were collected in early May (5/2/09 to 5/10/09) from three sites within approximately four square miles of the Carrizo Plain Ecological Reserve in San Luis Obispo County, California (see Chapter 1). In addition, two pregnant females were collected in early August of 2009 and held in the laboratory until parturition. A total of ten neonates (5 females and 5 males) were produced from the two litters.

Tissue Preparation and Measurement

Within 24 – 30 hours of capture, snakes were transported to the laboratory at California Polytechnic State University, deeply anesthetized via inhalation of isoflurane (Halocarbon Production Corps., USA), and perfused transcardially with a 0.9% NaCl, 0.1% $NaNO_2$, 0.1 M phosphate buffer (PB) wash at 10 ml/min for 10 min followed by 4% paraformaldehyde in 0.1 M PB, pH 7.2, with 0.1% $NaNO_2$ for 10 min. Skulls were removed and placed in 4% paraformaldehyde for at least 2 hours. Brains were then removed from the skull and post fixed for 24 hours in 4% paraformaldehyde. Next, brains were transferred to a 0.1 M PB solution for 24 hours prior to embedding in 8% gelatin. The gelatin was allowed to solidify overnight, then the embedded brains were placed into 4% paraformaldehyde for 24 hours and then into a 30% sucrose solution until they sank, at which time brains were frozen in dry ice and stored at -80° C.

Brains were sectioned coronally at 38 µm on a cryostat in four parallel series (Fig. 6). Free-floating sections were then stored in cryoprotectant at -20° C until mounting and staining procedures were performed. The first series was washed in PB solution, mounted onto slides with mounting solution, allowed to dry, and stained with cresyl violet. Brain sections were digitally photographed, and brain regions were measured using NIH ImageJ software. Measurements were taken by a single researcher blinded to snake sex. Medial cortex (MC), dorsal cortex (DC), lateral cortex (LC), and total telencephalon (TT) were identified as described in Halpern (1980). Measurements of cortical regions began once the LC cell layer was visible and continued throughout the telencephalon. The area of the telencephalon rostral to the LC cell layer was not included in the measurement of TT volume. Cortical subdivisions such as the large cell (dorsomedial) and small cell (medial) portions of the medial cortex were not considered separately, as described in Roth et al. (2006). To calculate regional brain volumes, area measurements from each section were added together and multiplied by the thickness of each section (0.038mm) then multiplied by 4 (the number of series). Volumes of both hemispheres were added to generate total volumes. Due to tissue damage during the mounting and staining procedure, DC and LC measurements are based on 8 individuals (4 males and 4 females). We mounted an additional series to further investigate relative MC volumes, so these data are from 6 females and 7 males. We only investigated MC volumes in neonates because this was the main region of interest.

Statistical Analysis

ANCOVA was used to test for sex differences in the volumes of MC, DC, and LC in adults and MC volumes of neonates. To control for variation in brain size among individuals, telencephalon volume was used as a covariate in all tests of cortical volume. As in Roth et al. (2006), telencephalon volume was quantified by subtracting cortical volume from total telencephalon volume on the corresponding brain slices. This technique provides a unique set of covariates for each brain region measured. We also included capture date and capture site in the ANCOVA model as random factors, but found that they were not significant ($F_{2, 4} = 1.1$, $p = 0.43$ and $F_{2, 4} = 0.3$, $p = 0.60$, respectively), and they were therefore taken out of the ANCOVA model. We used two-sample T-test to test for sex differences in overall telencephalon volume.

Results

The effect of the covariate telencephalon volume was significant in tests of DC ($F_{1, 13} = 31.8$, $p = 0.002$) and MC in adults ($F_{1, 13} = 13.6$, $p = 0.01$) and MC in neonates ($F_{1, 5} = 6.9$, $p = 0.05$), but not in tests of adult LC ($F_{1, 13} = 2.8$, $p = 0.15$). Although adult males had larger overall telencephalon volumes ($T_{11} = -2.88$, $p = 0.02$, Fig. 7), there were no sex differences in relative adult cortical volumes observed (DC: $F_{1, 13} = 1.3$, $p = 0.31$, Fig. 8; MC: $F_{1, 13} = 0.6$, $p = 0.52$ Fig. 9; LC: $F_{1, 13} = 0.4$, $p = 0.56$, Fig 10). Likewise, there was no significant difference in relative MC volume (ANCOVA: $F_{1, 4} = 0.45$, $p = 0.52$, Fig. 11) or overall telencephalon volume ($T_9 = 0.67$, $p = 0.53$, Fig. 12) between neonatal males and females. Mean values for brain region volumes are shown in Table 2.

Discussion

Despite the fact that male *C. o. oreganus* typically have larger home ranges and make more frequent and longer movements than females (Putman et al., in review), we did not detect any differences in relative MC, DC, or LC volumes between sexes and therefore failed to support the hypothesis that increased navigation drives sex differences in relative cortex volumes. However, although there was no sex differences in relative cortical volumes, adult male *C. o. oreganus* do have larger overall telencephalon volumes, which may allow for higher cognitive abilities as overall brain size as been positively correlated with mental ability in some animals. In humans, a larger overall brain size is correlated with a higher general mental ability (GMA) (reviewed in Rushton and Ankney 2009). Spearman (1904, 1927) found that overall brain size and GMA, which he dubbed "g" (general factor of intelligence), correlated positively with spatial, verbal, memory, and reasoning tests. Likewise, Deaner et al. (2007) reported that overall brain size was the best predictor of cognitive ability in non-human primates, suggesting that mental capacity and performance could be based on some absolute feature of the brain such as the combination of total number of cortical neurons and the conduction velocity of their fibers (Roth and Dicke 2005). However, this relationship has yet to be investigated in reptiles.

We predicted that adult male *C. o. oreganus* would have larger relative cortical volumes due to sex differences in home range size, but we failed to detect such differences in brain morphology. Our hypothesis is based on the fact that the MC has been demonstrated to be plastic in response to increased navigational demands over a

relatively short period in this species (Holding et al. 2012). Although we did not measure movements made by the snakes whose brain volumes are reported here, we did quantify the movements and home range sizes of another group of snakes of the same species, at the same field site, during the same time of year in 2009, and found that male snakes in 2009 had an average total distance moved of only 793.2 meters whereas male snakes in 2008 and 2010 averaged a total of 2046.3 and 1965.4 meters respectively. Likewise, male *C. o. oreganus* had smaller activity ranges as measured by 100% minimum convex polygons. That is, on average snakes in 2009 occupied 1.84 hectares whereas in 2008 and 2010 male snakes occupied 3.2 and 12.16 hectares respectively. Because snake movements appear to have been greatly diminished in spring 2009, perhaps due to the prolonged drought, male snakes may not have incurred an increase in their navigational demands at a magnitude necessary to elicit a change in cortical volumes. If this is the case, the lack of sex differences in navigational demands in 2009 may have led to the lack of differences in relative cortical brain regions, which could be in keeping with our hypothesis. Thus, male *C. o. oreganus* may only have larger cortical volumes than females during times when males are experiencing navigational demands beyond those of females. Our negative results could be a function of the variable nature and rapid plasticity of this character trait in addition to our data being taken in a year when sex differences in movement were likely very low. Furthermore, because the true differences between sexes due to navigational demands may be fairly small to start with (Roth et al. 2006), the detection of such differences could be very difficult if sex differences in navigation were diminished in 2009. Also, our small sample size makes detecting differences more difficult, especially if navigational demands were not as different

between sexes as they would be under non-drought conditions. Our small sample size could limit our ability to detect an effect of capture site on cortical volume which would add random error to cortical comparisons if there is a true, yet not detected, effect of capture site.

If cortical regions can increase rapidly in response to increased demands in *C. o. oreganus*, it is possible that these regions may also regress during times of low demands. This is likely the case as rapid growth and regression of brain regions in avian reptiles occur seasonally (reviewed in Meitzen and Thompson 2008). Thompson et al. (2007) showed that a song nucleus, HVC (acronym used as common name), regressed 22% within a 12 hour period after the removal of environmental breeding conditions (long photoperiod and systemic T exposure). Therefore, sex differences in cortical volumes of *C. o. oreganus* could be temporally associated with seasonal sex differences in navigational demands.

The mechanisms behind volumetric changes of cortical regions are unclear. Changes in the volume of specific brain regions could be attributed to changes in the number of neuronal or glial cells or their processes, or could be due to the increased distance between cell somas of brains regions (Nottebohm 1980). Additionally, changes in the amount of cell death could potentially affect volume of cortical regions. Kirn et al. (1994) reported seasonal changes in cell loss and recruitment within the HVC of male canaries, and that such changes were related to the seasonality of circulating hormone levels. Further experimentation could isolate the mechanism responsible for volumetric changes in cortical regions in rattlesnakes.

There was also no difference in MC volume between neonate male and female *C. o. oreganus*. This result is not surprising, as neonatal snakes born in captivity did not yet have to navigate, thus there were no navigational demands placed on them, and therefore no sex differences would be expected. To our knowledge, we report here the first data on MC volumes of neonatal reptiles. This is important because it demonstrates that sex differences do not exist at birth. Although other regions of the brain, such as the pre-optic area (POA), are known to be larger in male than female adult reptiles (Kabelik et al. 2006), it is unclear if this dimorphism develops pre- or postnatally. In birds, the sizes of the neurons within the POA appear to be irreversibly affected by embryonic steroids (Panzica et al. 1996); however, such data are not available for non-avian reptiles.

We suggest that changes in MC volume of *C. o. oreganus* are seasonal and occur over a relatively short time period. Results of this study highlight the need for further investigation into the timing and seasonality of volumetric changes in cortical regions of reptiles as well as the mechanisms behind such changes. Additionally, further investigation into the neurological morphology of neonatal reptiles is greatly needed and will likely benefit our understanding of the functional differences of the adult reptilian brain.

Table 1. Mean hormone concentrations and associated spatial parameters ± 1 SEM in free-ranging male Northern Pacific Rattlesnakes (*Crotalus o. oreganus*). Control snakes were implanted with silicone-filled implants and manipulated snakes were implanted with testosterone (T)-filled implants. Spatial parameters reported represent data collected from May 6 to June 29, which coincides with the period of time that T-implanted snakes had elevated T compared to control snakes.

Variable	**Control Snakes (n=10)**	**T-Treated Snakes (n=11)**
Testosterone (ng/ml)	**28.3 ± 4.1**	**109.6 ± 3.9**
Corticosterone (ng/ml)	**28.2±5.3**	**60.5±16.1**
100% MCP (ha)	**1.6±0.3**	**2.07±0.4**
95% MCP (ha)	**1.3±0.8**	**1.8±0.2**
95% Fixed Kernel (ha)	**0.3±0.2**	**0.5±0.1**
50% Fixed Kernel (ha)	**0.05±0.2**	**0.07±0.2**
Mean Distance Moved per Day (m)	**13.0±1.4**	**15.7±1.7**
Frequency. of movement	**0.5±0.2**	**0.5±0.2**
Mean Distance Moved Per Movement (m)	**76.0±7.1**	**89.0±5.4**
Total Distance Moved (m)	**714.5±83.3**	**858.7±94.4**
Maximum Movement (m)	**199.5±9.0**	**245.0±5.8**

Table 2. Mean volumes (mm^3 ± 1 SEM) of cortical regions and total telencephalon of adult and neonatal male and female *C. o. oreganus*.

Sex / Age Class	Medial Cortex	Dorsal Cortex	Lateral Cortex	Telencephalon
Adult Male	**9.71 ± 0.7**	**3.90 ± 0.4**	**2.87 ± 0.3**	**35.53 ± 3.4**
Adult Female	**8.23 ± 0.5**	**2.52 ± 0.5**	**2.47 ± 0.2**	**26.83 ± 4.9**
Neonatal Male	**2.74 ± 0.3**	**N/A**	**N/A**	**10.67 ± 0.9**
Neonatal Female	**2.52 ± 0.1**	**N/A**	**N/A**	**9.52 ± 0.5**

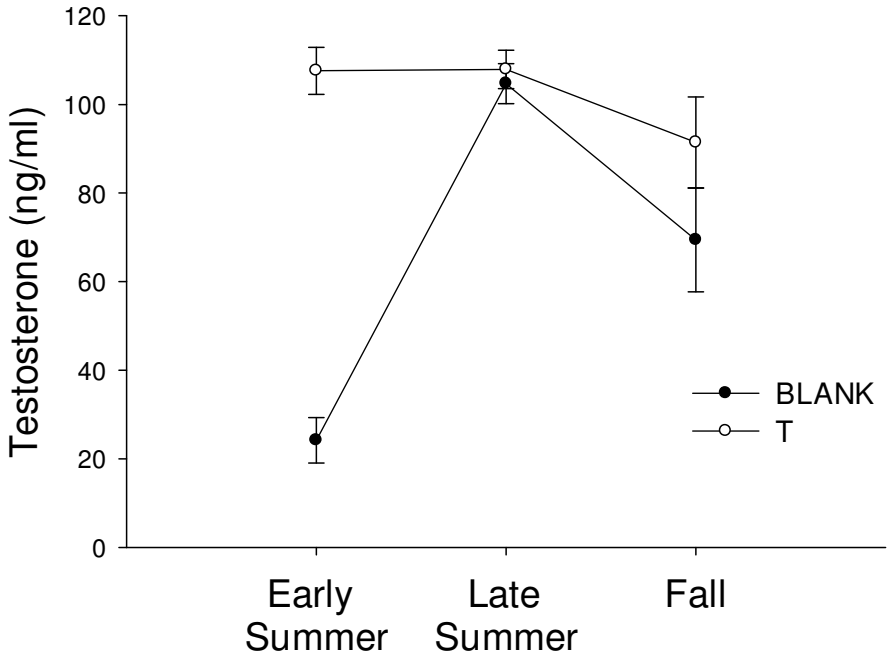

Figure 1. Mean plasma testosterone (T) concentrations (ng/ml +/- SEM) in control (N=10) and T-treated (N=11) male Northern Pacific Rattlesnakes (*Crotalus o. oreganus*) for three time intervals: Early Summer (May 29 - June 11), Late Summer (July 10 – 13), and Fall (August 26-Septermber 3).

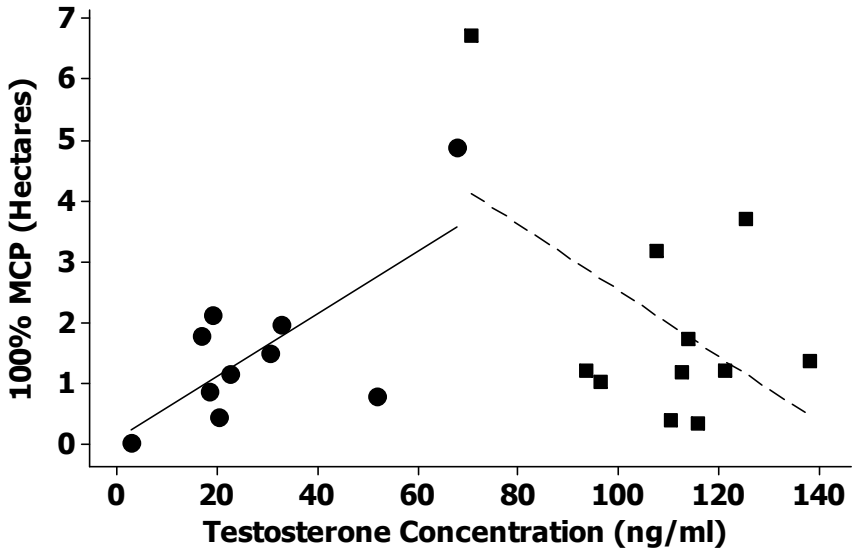

Figure 2. Relationship between plasma testosterone (ng/ml) and 100% Minimum Convex Polygon (MCP; Hectares) based on movement from May 6 to June 29, 2009 in control (circles; N=10) and T-treated (squares; N=11) male Northern Pacific Rattlesnakes (*Crotalus o. oreganus*).

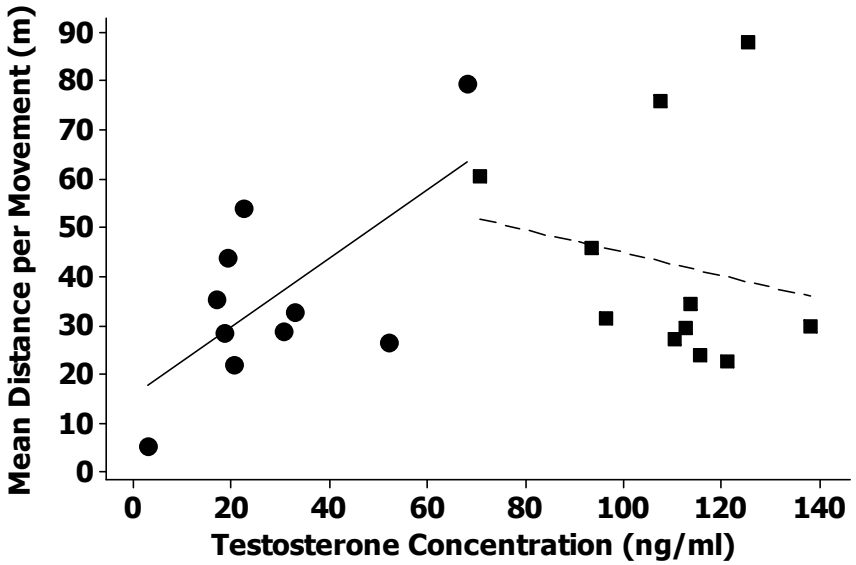

Figure 3. Relationship between plasma testosterone (ng/ml) and mean distance traveled per movement based on movement from May 6 to June 29, 2009 in control (circles; N=10) and T-treated (squares; N=11) male Northern Pacific Rattlesnakes (*Crotalus o. oreganus*).

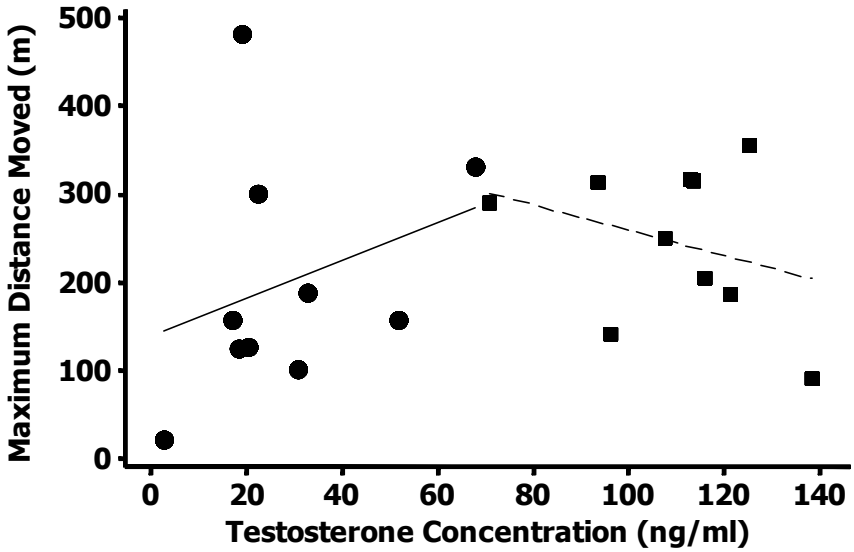

Figure 4. Relationship between plasma testosterone (ng/ml) maximum distance moved (meters) based on movement from May 6 to June 29, 2009 in control (circles; N=10) and T-treated (squares; N=11) male Northern Pacific Rattlesnakes (*Crotalus o. oreganus*).

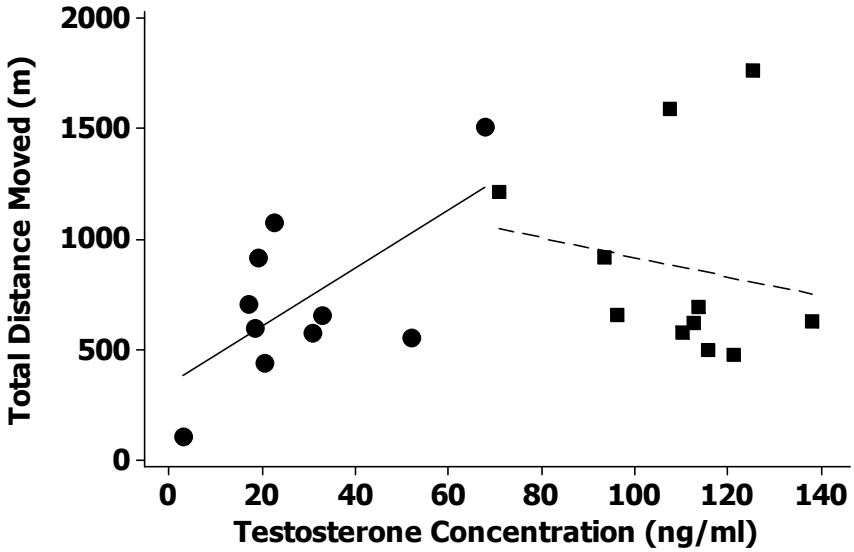

Figure 5. Relationship between plasma testosterone (ng/ml) and total distance moved based on movement from May 6 to June 29, 2009 in control (circles; N=10) and T-treated (squares; N=11) male Northern Pacific Rattlesnakes (*Crotalus o. oreganus*).

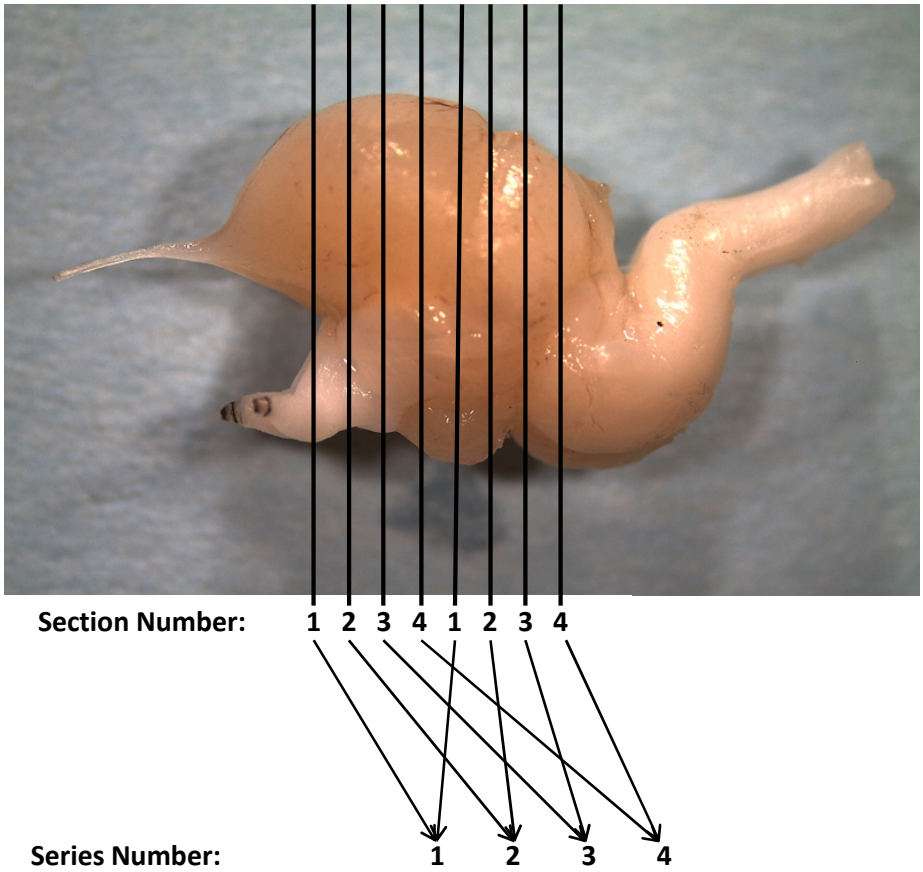

Figure 6**.** Schematic (lateral view) illustrating how individual parallel transverse sections through snake brains were organized into four series. Brain pictured above is a lizard brain and is solely for graphical representation of how sections were organized.

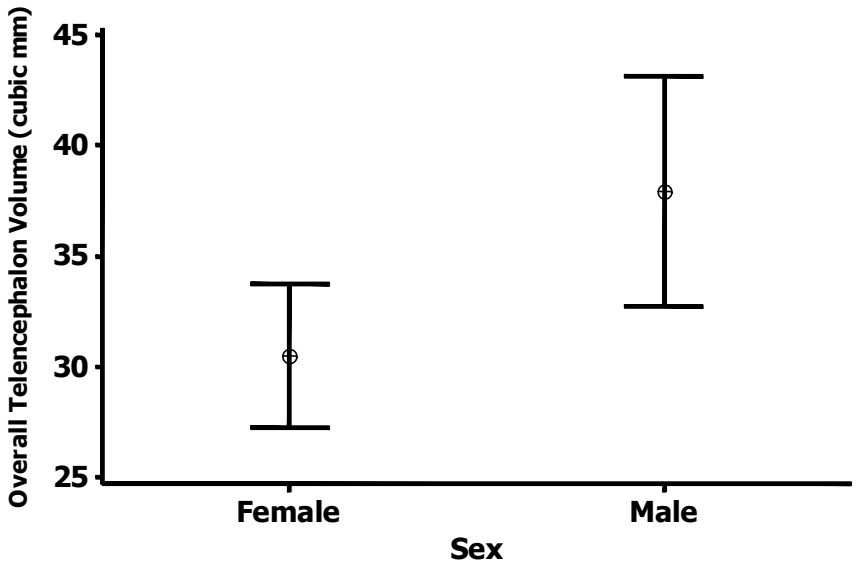

Figure 7. Overall telencephalon volume of adult female (N = 6) and male (N = 7) *C o. oreganus* collected in spring 2009. Male telencephalon volume is significantly larger than females (p = 0.02).

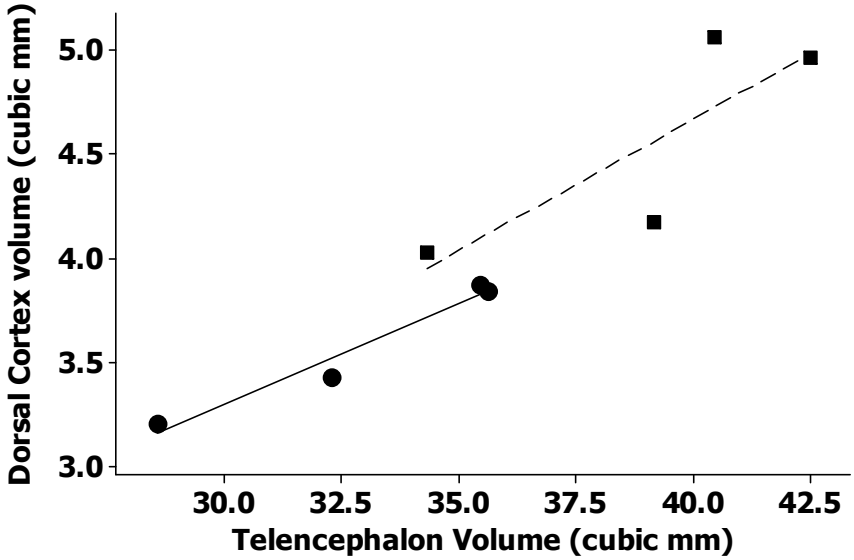

Figure 8. Dorsal cortex volumes of adult female (N = 4) and male (N = 4) *C o. oreganus* collected in spring 2009. Females are represented with circles and males with squares. No significant sex differences in relative dorsal cortex volume were observed (p = 0.31).

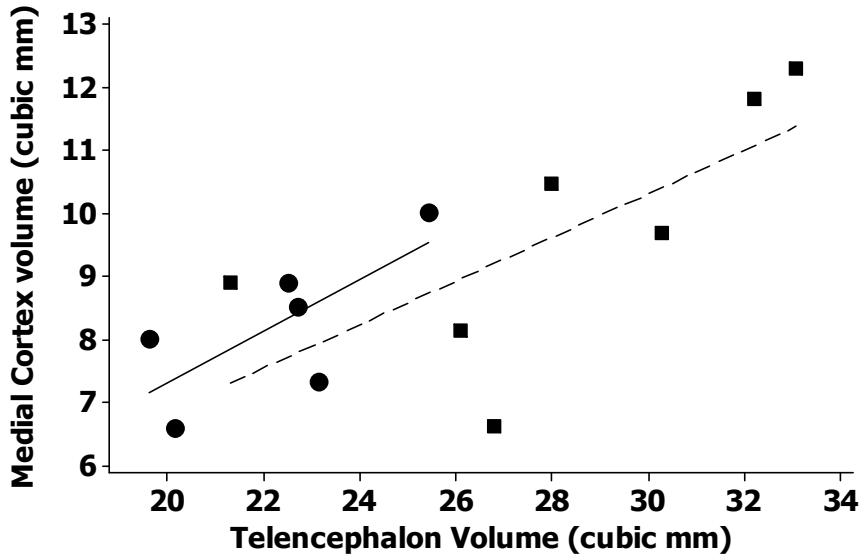

Figure 9. Medial cortex volumes of adult female (N = 6) and male (N = 7) *C o. oreganus* collected in spring 2009. Females are represented with circles and males with squares. No significant sex differences in medial cortex volumes were observed (p = 0.52).

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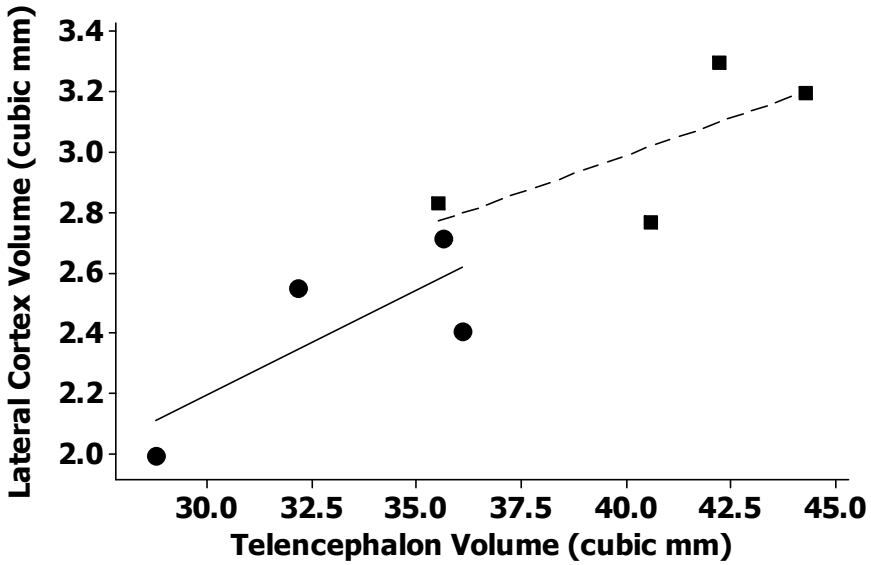

Figure 10. Lateral cortex volumes of adult female (N = 4) and male (N = 4) *C o. oreganus* collected in spring 2009. Females are represented with circles and males with squares. No significant sex differences in lateral cortex volumes were observed (p = 0.51).

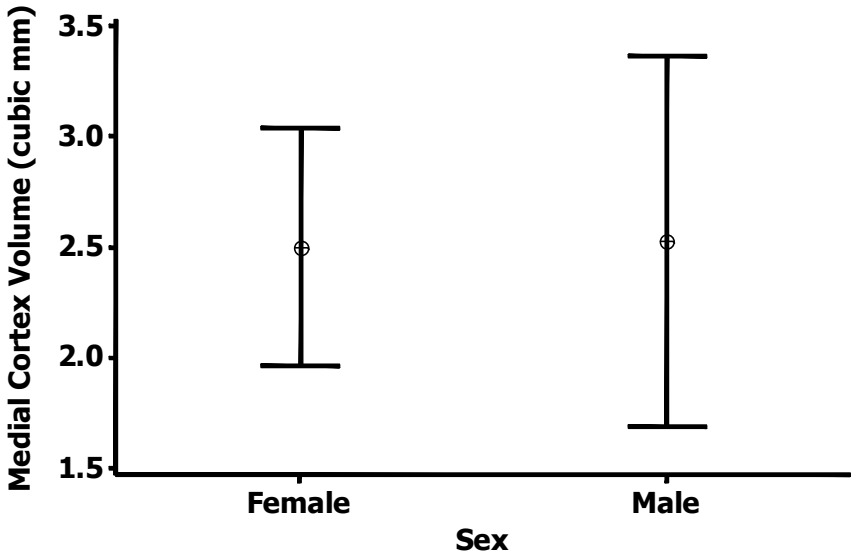

Figure 11. Medial cortex volumes of neonate female (N = 4) and male (N = 4) *C o. oreganus* born in the laboratory late spring 2009. No significant sex differences in medial cortex volumes were observed (p = 0.52).

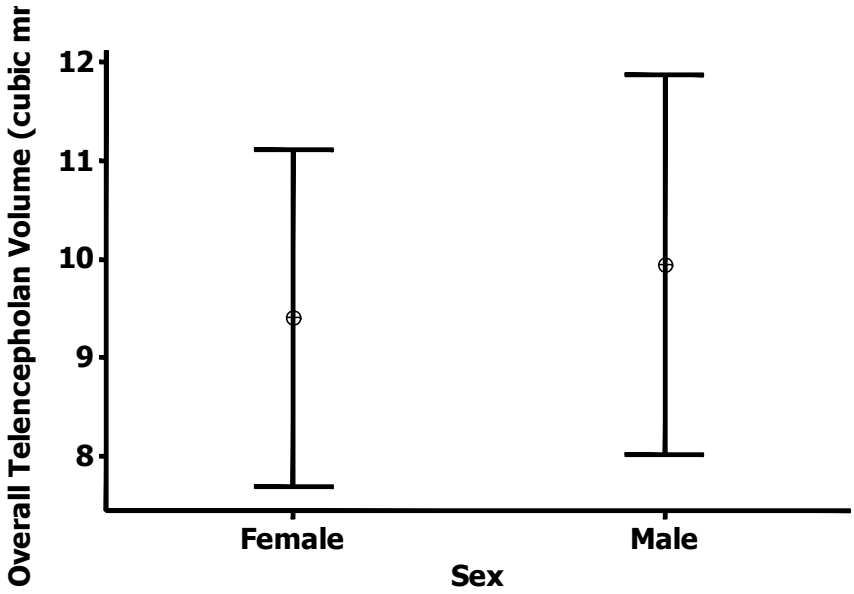

Figure 12. Overall telencephalon volume of neonatal female (N = 4) and male (N = 4) *C o. oreganus* born in the laboratory late spring 2009. No significant sex differences in overall telencephalon volumes were observed (p = 0.53).

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