TERRITORIAL BEHAVIOR AND CORTICAL BRAIN PLASTICITY IN ADULT MALE *SCELOPORUS OCCIDENTALIS*

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Daniel Robert Pfau

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AUTHOR: Daniel Robert Pfau

DATE SUBMITTED: April 2014

COMMITTEE CHAIR: Christine Strand, PhD
Associate Professor of Biological Sciences

COMMITTEE MEMBER: Emily Taylor, PhD
Associate Professor of Biological Sciences

COMMITTEE MEMBER: Gita Kolluru, PhD
Associate Professor of Biological Sciences

ABSTRACT

Territorial Behavior and Cortical Brain Plasticity in Adult Male *Sceloporus occidentalis*

Daniel Robert Pfau

The hippocampus is a brain region that can undergo tremendous plasticity in adulthood. The hippocampus is related to the formation of spatial memories in birds and mammals. In birds, plasticity in the hippocampus occurs when formation of such memories is directly relevant to survival or reproduction, such as for breeding or food caching. In reptiles, the homologues to the hippocampus are the dorsal and medial cortices (DC and MC). In several lizard, snake and turtle species, these structures have been related to spatial memory. Experimental investigations indicate that differences in DC volume are related to space use associated with differing foraging ecologies. Differences in MC volume have been associated with territory size-based mate acquisition strategies. Furthermore, territory size has previously been correlated with plasma testosterone (T) levels. Therefore, I hypothesized that neuroplasticity within the MC/DC is controlled by demands on spatial navigation and seasonal differences and that these changes may involve the action of T. During two experimental trials, male Western Fence Lizards (*Sceloporus occidentali*s) were placed into either large or small semi-natural enclosures and allowed to interact with a female and intruder males over the span of seven weeks. One trial was performed during the spring breeding season and the other during the summer non breeding season, to examine seasonal differences in plasticity. Blood samples were collected at initial time of capture and before sacrifice to measure plasma T. Immunostaining for doublecortin was used to determine the density of immature neurons in each region, and cresyl violet staining allowed for volume measurements of specific regions. MC cell layer neurogenesis was higher in lizards placed in large enclosures than those in small enclosures and higher in the summer than in the spring. DC volume was smaller in lizards held in large enclosures than those in small enclosures. The decreased DC volume seen lizards held in large enclosures may indicate a cost to the increased neurogenesis in the MC of lizards in the same enclosures. These results indicate a possible trade-off between DC volume and MC neurogenesis that allows for switching between the ability to solve novel spatial tasks using the DC while storing a cognitive map in the MC. During the spring, T had no relationship with MC volume, while during the summer this was negative, so effects of T on the MC may be seasonal.

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1. Introduction

Neuroplasticity is a phenomenon common throughout the lifespans of many organisms. This natural process occurs when the physiology and/or anatomy of the brain alters. Changes to the brain in this fashion are usually related to alterations in an organism's behavior. One well-documented system exhibiting neuroplasticity is the songbird song control system (Nottebohm, 1981). Song control nuclei grow and shrink through changes in the number, size, and/or density of neurons (Tramontin & Brenowitz, 2000). Some of these changes occur due to the addition of new neurons (neurogenesis) and the death of (apoptosis) neurons; these processes allow expression of seasonally specific behaviors, such as singing in males (Kirn et al., 1994). The expression of these seasonally relevant behaviors also has strong ties to changes in testosterone (T), an androgen produced at high levels during the breeding season (Nottebohm & Arnold, 1976; Small & Moore, 2009).

Well before the discovery and subsequent examination of neuroplasticity in songbird song control nuclei, Altman & Das (1965, 1967) discovered that neurogenesis occurs in the adult mammalian hippocampus. Despite these remarkable early discoveries, research on neuroplasticity in the hippocampus and in other vertebrates began in earnest during the 1990s, with advances in immunohistochemical techniques to identify newly born cells (Sherry & Jacobs,

1992; Clayton et al., 1997; Gould et al., 1999). Subsequently, the hippocampus has been established as a region that undergoes adult neurogenesis in many species, including humans (Maguire et al., 2000), rats and mice (Galea & McEwen, 2006; Birch et al., 2013), and several species of birds (Krebs et al., 1989; Sherry & Jacobs, 1992; Clayton et al., 1997).

Behaviorally, neuroplasticity in the hippocampus is related to spatial memory (Clayton et al., 1997; Maguire et al., 2000). For example, female Shiny Cowbirds (*Molothrus bonariensis*), a species where the female is the sole parent responsible for finding a nest to parasitize, have larger hippocampal regions than males. In a Cowbird species where both males and females search for nests, such as in the Screaming Cowbird (*Molothrus rufoaxillaris*), hippocampal volume is not sexually dimorphic (Clayton et al., 1997). Furthermore, activity involving heavy use of the hippocampus in humans—years employed as a London taxi cab driver—is correlated with an enlarged hippocampus (Maguire et al. 2000). Despite these relationships between spatial memory and hippocampal neuroplasticity, little is known about the mechanisms that cause these differences in neuroanatomy. Regulation of neurogenesis in the hippocampus may be related to excitatory neuronal activity (Lindsey & Tropepe, 2006) or effects of gonadal hormones (Galea et al., 2006).

The regions of the lizard telencephalon formed by the medial cortex (MC) and dorsal cortex (DC) are homologous to the hippocampus found in birds and mammals. Both these cortices show profound neuroplasticity in adulthood in many organisms and, like their homologues, have been functionally related to

spatial memory (Day et al., 2001; Ramirez-Castillejo et al., 2002; López et al., 2003; Holding et al., 2012). Differences in MC and DC volumes have been associated with season (Delgado-Gonzalez et al., 2008), territory strategy (LaDage et al., 2009), and foraging ecology (Day et al., 1999). In lizards, elevated levels of T during the breeding season have been associated with increases in territorial behavior, territory size, and variations in space use strategies. Differences in territory size or space use can lead to changes in spatial memory demands placed on individual lizards (Moore, 1987; 1988; Sinervo et al., 2000; LaDage et al., 2009). Supplementation of male Yarrow's Spiny Lizards (*Sceloporus jarrovi*) with T increased territory size acquisition above the already elevated levels during breeding season (Sinervo et al., 2000). Despite lizard T levels and cortical plasticity being associated with season and spatially relevant behaviors, no studies have examined the effects of T on the MC or DC. Only one study has directly manipulated spatial navigation demands in a reptile to examine the effects of spatial demands on the MC and DC (Holding et al., 2012).

Investigating comparative evolution of brain systems, such as the similarities among the MC, DC, and hippocampus, can uncover the ways in which nervous systems adapt to provide analogous functions in different groups of animals. Further exploration of the regulation of neuroplasticity in the MC and DC in reptiles will provide a comparative model with which to understand the evolution of neuroplasticity and the functional consequences of such neuroplasticity. Perhaps one of the most compelling reasons for research into

these reptilian brain regions is based on the findings of Molowny et al. (1995). They found that after lesioning the MC of European Wall Lizards (*Podacris hispanica*), a reactive neurogenesis within the MC could completely rebuild the structure, a phenomenon not demonstrated in the hippocampus of other vertebrates. Understanding the various ways organisms regulate neuroplasticity may provide insight into new treatments for human neurodegenerative diseases or nervous system injuries.

Here I present a study examining the associations among season, T, spatial navigation demands, and cortical neuroplasticity. In this chapter, I describe the pertinent research on the model organism used in this study, the Western Fence Lizard (*Sceloporus occidentalis*), and on the homology of the MC and DC of the reptile telencephalon to the hippocampus in mammals and birds, with emphasis on how neurogenesis occurs in these structures. The results from my study on the effects of season and territory size on plasticity and neurogenesis in the MC and DC of male *S. occidentalis* will be presented in chapter two. My study tests the hypothesis that territory size and season affect cortical neuroplasticity of the adult male *S. occidentalis* and that this may be mediated by T.

2. Model system: *Sceloporus occidentalis*

2.1 Territorial behavior

Sceloporus occidentalis is well known for its stereotyped territorial displays. Males perform territorial displays more often than females (Fitch, 1940). They can be observed displaying year-round but are especially aggressive during the breeding season of mid-April to mid-June (Fitch, 1940). Males will use these displays to ward off intruders in their territory, an area defended by a lizard for exclusive use. In contrast, a home range is the area inhabited by the lizard but not entirely defended for exclusive use, which may or may not be shared by multiple lizards (Sheldahl & Martins, 2000). The main territorial visual display is called a push-up, during which the lizard lowers its body by flexing its forearms then raises it again by extending its forearms (Fitch, 1940). This can vary based on the addition of several different body postures, including tail raising, lateral ventral flattening, and gular extending (Sheldahl & Martins, 2000). A full display or full show is defined as a lizard performing a push-up display while holding one of these body postures. Full displays are mainly used by fence lizards during their breeding season when they are at their most aggressive (Fitch, 1940, Sheldahl & Martins, 2000).

Male territories often overlap with one or more female territories during the breeding season (Sheldahl & Martins, 2000). In this way males “share” their territories with females while defending it from use by rival males. Survival of male lizards is dependent on their ability to defend sources of food and water. Even so, survival means nothing if they are unable to pass on their genetic

information and for this, they must also have access to females. If an intruder threatens to enter or has entered another male's territory, the resident male may display or become physically aggressive. When an attack occurs, the lizard will chase after the rival displaying territorial behaviors; most fights end after visual displays of aggression are exchanged. Rarely, lizards physically attack their rivals, chasing and biting at them or holding them in a bite. After an aggressive confrontation, the victor has solidified his claim to the space being fought over (Fitch, 1940). Territorial displays are recognizable from a large distance by even a casual observer, so they give researchers the ability to quantify the aggressiveness of individual lizards through simple observation.

In *S. occidentalis*, chemical signals are also very important in communicating with conspecifics (Duvall, 1979; 1981). Territorial *S. occidentalis* deposit chemicals from their femoral and proctodeal glands on subsrates, and this scent marking has been hypothesized to be as important as their visual displays. After a lizard senses the chemicals of another lizard, it will tongue flick the substrate (Duvall, 1979). This tongue flicking is thought to allow the use of their vomeronasal organ, which is located ventral to the main olfactory machinery and detects chemical signals from conspecifics (Duvall, 1979; 1981). An aggressive male will display a characteristic push-up pose after tongue flicking substrate that has exudates from another male (Duvall, 1979). Thus, the chemical signature of an intruder can cause males to display territorial behaviors, and the physical presence of the intruder is not required.

2.2 Hormones and spatial navigation/behavior

Conspecific chemical signals are not the only thing that can activate territorial behavior in lizards; hormones have a role to play as well. Increased territory size in side-blotched lizards (*Uta stansburiana*) has been positively associated with elevated T levels (Sinervo et al., 2000). It is unknown whether elevated T levels can improve the spatial abilities of lizards and the size of their territory or if the higher demands on spatial abilities due to a larger territory drives an increase in T levels. Testosterone has also been positively associated with increased territorial behavior in *S. jarrovi* (Moore, 1987). In both the closely related Eastern Fence lizard (*Sceloporus undulatus*) and *S. jarrovi* species, males have high T during the breeding season and low T during other times of the year (Moore, 1987; Klukowski & Nelson, 1998). Males also only show territorial behavior during the breeding season. During the other seasons, *S. jarrovi* males do not defend a territory, will tolerate intruders, and give very few, if any, territorial displays. This reduction in territorial behavior can be induced during the breeding season in *S. jarrovi* through castration, indicating the importance of T in activating territorial behavior (Moore, 1986; 1987). Additionally, supplementation of T in free-living, breeding-season *S. jarrovi* males caused an increase in female access and led to more overlap with female territories than previously observed (Moore & Marler, 1987). *Sceloporus undulatus* have peaks in T during the breeding season, which also coincide with increases in aggressive behavior (Mckinney & Marion, 1985; Klukowski & Nelson, 1998). *Sceloporus occidentalis* is considered to be at its most aggressive

and territorial during the breeding season as well (Fitch, 1940). There are no studies currently linking T to neuroplasticity within the MC or DC of lizards, but in male rats, the androgens T and dihydrotestosterone (a metabolite of T) have been associated with enhanced neuron survival within a portion of the hippocampus, the dentate gyrus (Spritzer & Galea, 2007).

2.3 Semi-natural enclosures

A semi-natural enclosure (as defined for purposes of this paper) is one that is exposed to the lizards' natural environment, but is still controlled in that it is an enclosed structure. In previous studies of *S. occidentalis*, researchers examined the social hierarchy of six to eight lizards in large enclosures (Tarr, 1976). The lizards in each enclosure were interacting in a similar way to those seen in wild populations. Semi-natural enclosures have also been implemented in experiments of aggressive behavior in the field. For example, Sheldahl & Martins (2000), placed *S. occidentalis* lizards into a small, mobile, semi-natural enclosure located at the site of capture. Using the arena as a staging area for behavioral trials, researchers observed the same stereotyped territorial responses to introduced conspecific males as occurred in a wild setting. These studies indicate that *S. occidentalis* continue to display and behave territorially when placed into a semi-natural enclosure.

Little is known about how the use of semi-natural enclosures affects physiology or endocrinology of lizards. In the Striped Plateau Lizard (*Sceloporus virgatus*), a lizard closely related to *S. occidentalis,* hormone levels of free-living

female *S. virgatus* did not differ significantly from those of females in captivity (Weiss et al., 2009). However, *S. occidentalis* kept in small, indoor enclosures had smaller MC & DC volumes compared to those who were living in the wild (Pfau et al., 2010). Thus, housing lizards in outdoor, semi-natural enclosures may reduce the negative effects of captivity on cortical brain regions.

3. Brain Regions Involved With Spatial Navigation

3.1 The hippocampus

Many studies have found the hippocampus to be involved with the spatial navigation abilities of an organism (Morris, 1981; Clayton & Krebs, 1994; Patel et al., 1997; Ghaem et al., 1997; Clayton et al., 1997; Maguire et al., 2006). Patel et al. (1997) demonstrated that formation of spatial memories in Zebra Finches (*Taeniopygia guttata*) declined in an object location task after lesioning of the hippocampus by ibotenic acid and the reversal of this effect through tissue transplant. For a nest-parasitizing Cowbird, spatial navigation is important in locating a host nest to parasitize. In *M. rufoaxillaris*, both the males and females search for a nest during the breeding season. They also have a larger hippocampus during the breeding season compared to other times of the year when spatial navigation is not as important (Clayton et al., 1997). In *M. bonariensis*, where the females are the sole parent responsible for finding the host nest, this seasonal change in hippocampal volume only occurs in the female brain (Clayton et al., 1997). In food-caching species, such as the Marsh Tit (*Parus palustris*), caching experience is positively associated both with

hippocampus volume and neuron number (Clayton, 1996). Interestingly, the hippocampus of birds with less caching experience had increased levels of cell death in the hippocampus (Clayton & Krebs, 1994). In birds, the hippocampus is used for spatial cognition, and plasticity is based on the level of spatial ability use.

The hippocampus is also involved with processing spatial memory in mammalian brains. Rats, even in the absence of visual, tactile, olfactory and auditory cues, are able to learn the location of objects in a maze (Morris, 1981). The ability to spatially navigate to find a goal object becomes impaired when the hippocampus is ablated through lesions, indicating the importance of this region in spatial navigation (Morris et al., 1982). Neuroplasticity related to spatial navigation within the mammalian hippocampus has been demonstrated in many species. For example, in Meadow Voles (*Microtus pennsylvanicus*), spatial navigation demands throughout the year are positively correlated with hippocampal neurogenesis (Galea & McEwen, 1999). Maguire et al. (2000) showed that the volume of the human right posterior hippocampus correlated positively with time spent as a London taxi operator, an occupation that requires the use of a previously learned cognitive map. The volume of the anterior hippocampus, on the other hand, was negatively correlated with time spent in this occupation. The posterior portion of the hippocampus seems to be involved with recall of already formed memories pertaining to spatial information (Ghaem et al., 1997), while the anterior portion of the hippocampus seems to be more involved with encoding of new spatial memories (Maguire et al., 2000).

Additionally, taxi drivers exhibited enhanced learned spatial abilities when compared to bus driver controls, yet showed reduced ability when solving novel spatial problems (Maguire et al., 2006). These results imply that the anterior hippocampus is involved with working in novel spatial situations, while the posterior portion assists in cognition of learned spatial environments; further, enhancement of one of these regions and abilities comes at a cost to the other.

3.2 Cross species neuroanatomical comparison

In general, the mammalian hippocampus is found medially within each of the cerebral hemispheres and is surrounded by the neocortex. It begins medial within the cortex and then follows the lateral ventricle posteriorly and laterally. The avian hippocampus is superficial and medial within the posterior portion of the cerebral hemispheres, following the lateral ventricles (Butler & Hodos, 2005). The homologue to the hippocampus in reptiles is found in a superficial portion of the reptilian cortex, superior to the lateral ventricles. There are three main regions within the reptilian cortex, the medial cortex (MC), dorsal cortex (DC) and lateral cortex (LC; Figure 1). Anterograde tract tracing of neurons in the olfactory bulbs of the *P. hispanica* demonstrated that the LC has more similarity to the mammalian olfactory cortex and is not directly involved with spatial functions (Martinez-Garcia et al., 1986). The dorsal cortex has circuitry shared with the medial cortex that resembles that of limbic system structures in mammals. Within the medial cortex there are three layers of cells, with the majority of cell soma lying in the cell layer (CL). The dendrites of these neurons mainly lie in the outer

plexiform layer (OPL) where there are few neuron cell bodies. The neurons of the CL receive projections from multiple locations in the telencephalon. The inner plexiform layer (IPL) is comprised of efferent and afferent projections of the CL and cells moving from the sulcus septomedialis, along the lateral ventricles, into the cell layer, there are also a few soma that reside in the IPL. The DC is structured similarly to the MC, although the neurons in its cell layer mainly send and receive messages between itself and the MC (Lohman & Smeets, 1991; Butler & Hodos, 2005). Together, the MC and DC form the homologue to the more complex mammalian and avian hippocampus (Butler & Hodos, 2005).

The MC is most similar to the dentate gyrus of the mammalian hippocampus. The neurons in the MC CL have zinc-rich axons and seem to have a similar cytoarchitecture as the mossy fibers found in the mammalian dentate gyrus (Martinez-Guijarro et al., 1991). The presence of GABA neurons in the dorsomedial cortex (a subregion of the MC) suggests a function similar to that of Ammon's horn of the mammalian hippocampus (Butler & Hodos, 2005). These comparisons have led to several studies into the relationship between the reptile cortex and spatial cognition (Font et al., 1991; Day et al., 2001; Holding et al., 2012).

4. The Reptilian Cortex

4.1 The cortex region and spatial cognition

Changes in behavior brought on by lesions to the MC and DC have demonstrated that, like the hippocampus, the cortex is involved with spatial learning, memory, and other non-spatial tasks (Font et al., 1991). In Scheltopusik (*Ophisaurus apodus*), lesions of the MC have led to the loss of conditioned responses involving spatial memory and interfere with reversal learning (Ivazov, 1983). Lesions in both the MC and DC lead to increased latencies to finding a goal rock in the Little Striped Whiptail (*Cnemidophorus inornatus*) (Day et al., 2001). In the Pond Slider Turtle (*Pseudemys scripta*), learned place-task performance, an indicator of spatial abilities, was impaired by MC lesioning (López et al., 2003). Furthermore, in Northern Pacific Rattlesnakes (*Crotalus oreganus oreganus*), experimentally increasing spatial demands through translocation caused MC volume to increase (Holding et al., 2012). These studies emphasize the importance of the MC and DC for spatial reasoning of reptiles and indicate their potential for neuroplasticity.

4.2 Cortex region hormonal interactions

The interplay of neuroplasticity and hormone signaling that occurs between the male songbird song control system and T is one of the well-studied examples of these interactions (Smith et al., 1997a; 1997b). Studies in Gambel's White-crowned Sparrows (*Zonotrichia leucophrys*), Song sparrows (*Melospiza melodia*), and Dark-eyed Juncos (*Junco Hyemalis*) all indicate T as the primary

signal leading to seasonal changes of the song control nuclei. These seasonal changes in hormones and neuroanatomy are also related to cycles of singing behavior between seasons (Smith et al., 1997a; 1997b; Dloniak & Deviche, 2001), much like the seasonal increases in territorial behavior seen in *S. occidentalis*. In lizards, the correlation between circulating T levels and territory size (Sinervo et al., 2000) along with the connection between T and territorial behavior (Klukowski & Nelson, 1998; Sheldahl, 2000) implicate T as a major factor affecting brain regions involved in spatial navigation related to maintaining a territory during the breeding season. Indeed, in another lizard, the Green Anole (*Anolis carolinensis*), androgen receptor (AR) and AR messenger RNA are present in cells in the cortex region (Rosen et al., 2002). It is likely that, similar to the song control nuclei of birds, plasticity of the lizard cortex may be influenced by blood plasma T concentrations.

4.3 Cortex region plasticity

Neuronal proliferation in the cortex is of particular interest, as there are many examples of postnatal neurogenesis within the cortex region of lizards (Molowny et al., 1995; Ramirez et al., 1997; Font et al., 2002; Delgado-Gonzalez et al., 2008). Additionally, after chemical ablation with the toxin 3-acetylpyridine (3-AP), the MC goes through a reactive neurogenesis process to produce a newly formed MC indistinguishable from those that had been unaltered (Font et al., 1991; Molowny et al., 1995). When lizards are exposed to low temperature and fewer hours of light, the reactive neurogenesis following 3-AP lesioning does

not occur. Neuroplasticity in this reptilian cortex is therefore affected by changes in photoperiod and temperature (Ramirez et al., 1997). Additionally, captivity was shown to be detrimental to neurogenesis in all seasons except autumn, when neurogenesis levels are already very low (Delgado-Gonzalez et al., 2008).

The cortices of wild lizards with varying spatial navigation behaviors and demands have also shown variation in the amount of plasticity present in these spatially related neural structures. The MC and DC are larger in congeneric lizards that have an active foraging strategy, Bosc's Fringe-toed Lizard (*Acanthodactylus boskianus*), than in a sit-and-wait congeneric, Nidua's Fringe-toed Lizard (*Acanthodactylus scutellatus*; Day et al., 1999). Active foragers have higher navigational demands, as they must go out and search for prey while sit-and-wait predators need only stay in a few locations for long periods of time. In male Side-blotched Lizards (*Uta stansburiana*), the DC is larger in those males that hold and maintain territories than in those that do not keep one (LaDage et al., 2009). These differences suggest that the increased demand for spatial memory and learning may have a connection to their plasticity.

I conducted an experiment to examine the regulation of cortical neuroplasticity in lizards through experimental manipulation of male lizard territory size by placing them in semi-natural enclosures of two sizes. Based on the strong connections between neuroplasticity and season in the songbird song control system (Nottebohm, 2002; Ball & Balthazart, 2010), I also examined seasonal variation in neuroplasticity by repeating the experiment during the spring breeding season and the summer nonbreeding season. Finally, based on

T modulation of the songbird song control system (Dloniak & Deviche, 2001; Nottebohm, 2002; Ball & Balthazart, 2010) and its seasonal patterns in lizards (Klukowski & Nelson, 1998; Pollock et al., 2012), I examined the possible effects of T on cortical neuroplasticity. I hypothesized that neuroplasticity within the neuroplasticity within the medial and dorsal cortex of the lizard telencephalon would be mediated by season and territory size through the action of T.

Chapter 2: Environmental and Hormonal Regulation of Cortical Plasticity and Behavior in Male *Sceloporus occidentalis*.

1 Introduction

During development, the brain remains plastic as it goes through changes leading to the development of a mature organism (Knudsen, 2004). Many of these changes are important for the performance of behaviors vital to an organism's survival and fecundity, such as activation of adult sexual behaviors (Mohr & Sisk, 2013). Recent examinations into the adult brain have indicated that even after developmental periods have ended, adult neuroplasticity continues and can lead to profound changes in behavior (Pravosudov & Omanska, 2005; Klempin & Kempermann, 2007; Delgado-Gonzalez et al., 2008). One salient example of adult neuroplasticity is that of the avian song control nuclei (Nottebohm, 1981). Changes to an organism's external (photoperiod, temperature, spatial demands, etc.) and/or internal (hormone levels, reproductive state, etc.) environment can cause growth or regression of the song control nuclei (Gulledge & Deviche, 1998; Dloniak & Deviche, 2001; Strand & Deviche, 2007; Ball & Balthazart, 2010).

Adult male songbird song control regions are highly plastic and the relationship between internal and external cues, neuroplasticity, and behavior has been outlined in great detail (Kirn et al., 1994; Nottebohm, 1981; 2002). Yearly patterns of song control nuclei neuroplasticity follow seasonal patterns in adult sexual behavior. Changes in the external environment, such as increasing

day length in the spring, can stimulate the hypothalamic-pituitary-gonadal axis changes internally and lead to the activation or termination of reproductive behaviors (Smith et al., 1997a, 1997b). These external and internal changes mediate behavior by manipulating neuroplasticity in the song control regions of the brain (Nottebohm, 2002). Testosterone levels rise during the breeding season which slows the rate of apoptosis in the HVC. This increase in neuron lifespan leads to larger numbers of neurons in the HVC where they can induce changes to behavior such as the seasonal increases in song. After the breeding season, T levels drop and apoptosis increases within the HVC which attenuates male songbird singing behavior (Rasika et al., 1994; Kirn et al., 1994; Nottebohm, 2002; Strand & Deviche, 2007). The neuroplasticity within the song control nuclei is therefore mediated by seasonal and hormonal changes, and these changes in the brain can lead to changes in behavior.

Another region that shows adult neuroplasticity in birds and mammals is the hippocampus, a region involved with spatial cognition (Krebs et al., 1989; Sherry et al., 1992; Gaulin, 1992; Clayton et al., 1998; Maguire et al., 2000; Lindsey & Tropepe, 2006). Spatial navigation demands can vary between species based on different parenting strategies or foraging ecology. Within species, spatial navigation demands can also differ due to diverse mating strategies or, in humans, even occupation. Differing nesting strategies have been used to explain sexual dimorphism in the size of the cowbird hippocampus (Clayton et al., 1998). Furthermore, in human taxi drivers, a spatially demanding job, the size of the hippocampus correlates positively with time spent in this

career and (Maguire et al., 2000). Together, these studies indicate that increased spatial navigation demands in the environment are associated with an increase in hippocampus volume.

Still other environmental factors are known to affect neuroplasticity in other regions of the brain. One such factor is the complexity of the space an animal occupies in captivity. The introduction of running wheels, new toys, novel social interactions, and more cage space (collectively known as environmental enrichment) all cause increases in neurogenesis compared to an empty cage (Kempermann et al., 2002; Tanti et al., 2013). The increases in neurogenesis due to environmental enrichment are independent from changes in exercise (Birch et al., 2013) and have been put forth as a possible treatment for cognitive impairments by enhancing neurogenesis (Fares et al., 2013). Furthermore, in birds and mammals, spatial experience and stimulation are involved with the regulation of neuroplasticity in the hippocampus (Clayton et al., 1994; Maguire et al., 2006). In food-caching birds, hippocampal volumes are positively associated with increased food-caching activity, a taxing experience for spatial abilities. While increases to caching experience led to enhanced neurogenesis and hippocampal volume, decreased caching experience was marked by lower levels of neurogenesis and smaller hippocampal volumes (Clayton & Krebs, 1994; Clayton, 1996). This suggests a mechanism of “use it or lose it” in which use or excitatory neuronal activity of an area is necessary to maintain or promote neuron survival or growth.

The cortex region in reptiles is thought to be a homologue of the hippocampal formations in birds and mammals (Martinez-Guijarro, 1991; Dávila et.al., 1993; Day, 2001). Specifically, the medial cortex (MC) and dorsal cortex (DC) are involved with hippocampal-like functions, such as spatial memory (Day, 2001) while the other cortical region, the lateral cortex (LC), is involved with olfaction (Martinez-Garcia et al., 1986). In reptiles, the MC and DC have very similar circuitry (Lopez-Garcia & Martinez-Guijarro, 1988), cytoarchitecture (Martinez-Guijarro, 1991), and neuronal life history (Dávila et.al., 1993) as the hippocampus. These cortical regions have already been lauded as important models through which the mechanisms of action in the more complex and interconnected avian and mammalian hippocampus may be examined (Lopez-Garcia et al., 2002).

As with the hippocampus of birds and mammals, the MC and DC of reptiles are involved with spatial memory and cognition. Lesions of the MC and DC impair spatial abilities in lizards and turtles (Day et al., 2001; López et al., 2003). Differences in the volume of the reptile MC and DC have been correlated with variations in several behaviors including foraging ecology (Day et al., 1999) and territory size based on mate acquisition strategies (LaDage et al., 2009) in the wild. In free living Northern Pacific Rattlesnakes (*Crotalus oreganus oreganus*), a causal relationship was discovered between MC volume and experimentally altered demands on spatial abilities (Holding et al., 2012). Furthermore, captivity in a non-stimulating and confining environment decreases MC volume (Pfau et al., 2010).

Similar to the singing behavior of birds, territorial behavior in lizards is likely seasonally modulated by photoperiod and temperature control of testosterone levels (Fitch, 1940; Marion, 1982; Moore, 1988; Klukowski & Nelson, 1998). The stereotyped territorial behaviors of the model species used in this study, the Western Fence Lizard (*Sceloporus occidentalis*), have been well documented in several studies (Fitch, 1940; Duvall, 1979; 1981; Sheldahl & Martins, 2000). *Sceloporus occidentalis* males are more aggressive than females, and are especially prone to display these behaviors during the breeding season of mid-April to mid-June (Fitch, 1940). In a closely related species, lizards with higher levels of T, such as a male during the breeding season, are known to be more aggressive in some populations of Eastern Fence Lizards (*Sceloporus undulatus;* Klukowski & Nelson, 1998). In many lizard species, each male has a territory over which he maintains exclusive use. This territory is also part of a larger area called the home range, which is a region of the environment he visits but does not actively defend as his own. A territorial male *S. occidentalis* displays characteristic push-ups often accompanied by several body postures to defend his territory (Sheldahl & Martins, 2000) and in another species, *U. Stansburiana,* high T can increase the probability that a lizard will aggressively respond in this way (Sinervo et al., 2000). Thus, there is a possible connection between T and an organism's ability to acquire or maintain a territory. Interestingly, neurogenesis in the MC and DC of the Tenerife lizard (*Gallotia galloti*) is at its highest during its mating season, the spring (Delgado-Gonzalez et al., 2008). Spring is also the mating season for *S. occidentalis* and a time of the year that is marked by

elevated T levels and territorial behavior in males (Fitch, 1940; Pollock et al., 2012). Furthermore, lizards with larger territories have higher T levels than those with smaller territories (Sinervo et al., 2000). Photoperiod and temperature modulate neuroplasticity in the reptilian cortex (Ramirez et al., 1997), but it is unknown if these effects are mediated by changes in T (Molowny et al., 1995; Ramirez-Castillejo et al., 2002). Therefore, reptile cortical neuroplasticity is likely regulated by season, hormone, or stimulation based on spatial navigation demands.

In the present study, I hypothesized that neuroplasticity within the MC and DC is regulated by territory size and season and is mediated through the actions of T. Through the use of semi-natural enclosures, the size of a lizard's territory was manipulated, and by holding two trials, one in spring and the other in summer, I examined the effects of T and season on cortical neuroplasticity.

2 Materials and Methods

2.1 Treatments

Adult male and female *S. occidentalis* were collected from Poly Canyon, San Luis Obispo (Latitude: 35.31/ longitude: -120.67) during the week of April 25, 2011 (n=14, breeding season) and the week of June 27, 2011(n=14, non-breeding season) (Davis, 1967). Collecting permits were obtained through the California Department of Fish and Game and all procedures were completed with approval by the Cal Poly Institutional Animal Care and Use Committee. Using modified fishing poles, wild lizards were noosed around the neck using waxed

fishing line. Each lizard was assigned an experiment number which was marked on the lizards through toe clips (Dunham et al., 1988) and painted on their backs with Wite-out ® (BIC inc.) for easy identification. Blood was collected from the retro-orbital sinus of experimental males within 5 minutes of them being caught. Blood samples were kept on ice, transported to the lab, centrifuged, and plasma was collected. Plasma samples were frozen and stored at -80°C. Lizards were transported individually in cotton sacks to our lab facilities. Snout-vent length (SVL) and mass were both recorded in the laboratory and lizards were transported to the site of their enclosures (see below). Female and male lizards were randomly paired and placed into large or small resident enclosures. A separate group of intruder males (n=14) was captured from the same field site and housed together in a large enclosure.

Enclosures were constructed in an agriculture field on the Cal Poly campus (Latitude: 35.31/ longitude: -120.68), less than 5 km away from the location where the lizards were captured. Both large (5.95 m^2; 2.44 m x 2.44 m, n=7 for each season) and small (1.46 m^2; 1.21 m x 1.21 m, n=7 for each season) enclosures were made from 0.6 m tall, 1.27 cm thick white opaque corrugated plastic sheets. Large enclosures contained four cinderblocks, three water dishes, and eight artificial plants, while small enclosures contained one cinderblock, one water dish, and two artificial plants. Each enclosure was filled with a one inch-deep layer of woodchips along with a large pile on the south-facing wall of the enclosure to allow for burrowing. Lizards were given water *ad libitum* and fed crickets every other day. Between spring and summer trials, all plants,

cinderblocks, and water dishes were washed with water then placed in the sun for two weeks. The woodchips were removed and the enclosures themselves remained empty and exposed for a period of two weeks before new woodchips and the washed cinderblocks, plants, and water dishes were replaced.

2.2 Behavioral testing

Each resident male was given at least three days to acclimate to the enclosures before behavioral testing began. Behavioral tests for the breeding season began on April 30, 2011 and continued for seven weeks until June 11, 2011. For the non-breeding season, behavioral tests began on July 11, 2011 and continued for seven weeks until August 23, 2011. During both the breeding season and non-breeding season, all behavioral testing was completed between the hours of 10:00 and 17:00, weather permitting. The order in which each enclosure was tested was randomized each day so that a new order was used for each day of behavioral observations. Each trial involved using intruder males to elicit aggressive responses from resident males. An intruder male was chosen haphazardly from the intruder enclosure, and then placed on a one foot tether at the end of a modified fishing pole. Each intruder was used for five separate behavioral trials or until they were physically attacked by a resident. At the start of each trial the location of the resident male was determined. If the resident male was visible, the intruder was placed in a location visible to him. After one minute, if no interactions had been noted by observers, the intruder was moved to another location still in the view of the resident male. Trials during which the

resident was not seen lasted for five minutes; observers changed the location of the intruder each minute provided an interaction had not begun. Trials that included an interaction were allowed to run for the full six minutes unless there was a physical attack by the resident or intruder, in which case the trial was ended and the time to attack was noted. If the resident male was not visible, the intruder males were placed in five separate locations for one minute each for a total of five minutes spent in the enclosure. This was done to maximize the probability that the resident male would see the intruder and begin an interaction. If the resident male appeared during a trial, the trial was continued for the full six minutes. After being used for behavioral trials the intruder male was returned to its enclosure. At that point it would no longer be used for a trial until all other intruder males had been selected and used. Haphazard selection from the pool of usable intruder males was performed until all males had gone through behavioral trials. At this point, all males were allowed to be used again and haphazard selection began again on the entire population of intruder males. In this way, the same intruder would not be used again until all other intruder males had been selected. Behavioral trials were carried out four times a week for seven weeks.

During behavioral testing, the numbers of aggressive and submissive behaviors of the resident male were measured. Aggressive displays included several behaviors. Push-up displays were counted when the resident male would flex his limbs lowering himself to the ground then extending them again to push-up. Each pair of lowering and raising was counted as one display. Lateral-ventral

(LV) compressions occurred when the male flattened his body along the midsagittal plane, exposing his brightly colored ventral surface. Males also performed gular extensions of the throat and tail raises. Full displays or shows were considered a push-up display done in conjunction with an LV compression, gular extension, and/or tail raise (Sheldahl, 2000). A chase was considered any time the resident male rapidly moved toward and within one inch of the intruder male. Bites were recorded anytime the resident male opened his mouth and placed it on the intruder regardless of whether he held on or not. Anytime there was a bite the trial was ended and the latency to bite was noted. After a bite, the intruder male was removed to prevent any physical harm and released back into the intruder enclosure so the physical altercation would not affect the results of any subsequent behavioral trials. For each resident male lizard, the total number of push-up displays, full shows, chases, and bites were determined. Lizards were also categorized as “interacting” or “non-interacting” based on whether or not they performed any aggressive displays towards intruders during the behavioral assays.

2.3 Sacrifice and Immunohistochemistry

At the end of the 7-week behavioral testing period, resident males were removed from their enclosures, a blood sample was collected immediately (as previously described), and lizards were placed into buckets and transported back to the lab. Females and intruder males were removed from their enclosures and released back to the locations they were originally captured. SVL and mass were

measured for each resident male before sacrifice through cardiac perfusion under anesthesia. Once deeply anesthetized through isoflurane inhalation, lizards were transcardially perfused with a 0.1M phosphate buffer (PB), 0.9% sodium chloride, and 0.1% sodium nitrite wash. This was followed by 4% paraformaldehyde in 0.1M PB with 0.1% sodium nitrite. Brains were removed and postfixed in paraformaldehyde overnight and then placed in 0.1M phosphate buffer after which brains were weighed and trimmed. Then, the brains were embedded in 8% gelatin. The gelatin was allowed to set for 24 hours, and each gelatin-encased brain was then trimmed and placed in 4% paraformaldehyde overnight. Twenty four hours later the brains were transferred to a 30% sucrose solution until they sank (approximately 48 hours) at which point they were placed in dry ice to be stored at -80° C.

Brains were sectioned into three series of parallel transverse sections at a thickness of 40 µm using an H/I Bright cryostat held at -20°C. One series was mounted directly onto slides and stained with cresyl violet. The other two series were placed in cryoprotectant and stored at -20°C. Cresyl violet stained sections were photographed on a Leica light microscope. Image J software was used to measure the area of the MC and DC (Figure 1) on each brain section as well as the total telencephalon area to control for brain size (Day et al., 1999). A second series was used to stain for doublecortin (DCX), a marker for immature neurons and used as an indicator of the level of neurogenesis. Sections were blocked in a 5% normal horse serum/0.5% hydrogen peroxide solution in phosphate buffer, then incubated with DCX antibody (1:4000, Santa Cruz Biotechnology) in

phosphate-buffered saline (PBS) with 0.3% Triton X-100 overnight at room temperature on a rotating table. They were then incubated with biotinylated anti-goat secondary antibody (1:100, Vector Laboratories, Inc, Burlingame, CA) in phosphate-buffered saline with 0.3% Triton X-100, followed by treatment with ABC solution (Vector Laboratories, Inc.) and finally Vector SG chromagen (Vector laboratories, Inc.). Stained sections were photographed on an Olympus BX-60 light microscope, and DCX-immunoreactive (DCX-ir) cell numbers were counted in the inner plexiform layer (IPL) and cell layer (CL) of the MC in every 3rd section through the telencephalon. DCX-immunoreactive cells were not present or present at only small amounts in outer plexiform layer of the MC and in the entire DC, so data were not collected or analyzed for these areas. The volume of each of these regions was determined using Image J software as described above, and the density of DCX-ir cells was determined for each region. During measurements of volume and counts of DCX-ir cells researchers were blind to treatments.

2.4 Radioimmunoassay

Radioimmunoassay (RIA) was performed on the blood plasma samples collected in the field and at the end of behavioral testing. All samples were randomly assigned to assays and T concentration levels were assayed using an established protocol previously described in Moore (1986). 30% ethyl acetate/isooctane extractions were performed then separated, dried, and resuspended in 10% ethyl acetate in isooctane. Testosterone was separated

from samples in a 20% elution of ethyl acetate/isooctane. Samples were collected in vials, dried, and resuspended in PBS buffer. A duplicate aliquot of each sample was assayed for T levels. The intra-assay coefficient of variation was 0.066%.

2.5 Data analysis

Testosterone data were log-transformed and a two-way repeated measures ANOVA was used to compare T levels between the seasons and time of collection (at the beginning and end of each season) then post hoc analysis with Holm-Sidak was performed for multiple comparisons using SigmaPlot 12.5 (Systat software, Inc.). Testosterone levels were predicted to be elevated during spring and at low levels during summer.

To examine the difference in the number of interacting lizards between seasons, a chi-square analysis on Minitab 16 Statistical Software (Minitab Inc.) was performed. To determine if there was a seasonal difference in lizard activity, measured by number of lizards marked as being visible by the researcher during behavior trials, an ANCOVA was performed with SVL and blood plasma T concentrations as covariates. To determine a difference in lizard activity between enclosure sizes, another ANCOVA was run comparing the number of lizards visible during behavioral trials in large enclosures to those in the small. SVL and blood plasma T concentrations as covariates. The total numbers of aggressive displays per individual were inverse-transformed, and then an ANCOVA was used to determine differences between seasons (spring vs. summer) and

treatment (larger vs. small) using SVL and blood plasma T concentrations as covariates. Descriptive statistics were also performed to facilitate discussion on behavior. I predicted lizards from spring would be more likely to aggressively interact as compared to the lizards from summer.

For all volumetric analyses, regressions of total telencephalon volume (excluding the region being tested) and the volume of the region being tested were performed and the standardized residuals from regressions were used for analysis (Day et al., 1999). This was done to control for differences in individual brain sizes. ANOVA was performed using enclosure size and season as fixed variables and enclosure location as a random variable on Minitab 16 Statistical Software. Tukey's post-hoc analyses were performed if main effects or interactions provided significant results. I predicted greater volumes for the MC, DC, and MC CL of lizards held in large enclosures compared to small. I also predicted greater volumes for these regions in lizards held during the spring season.

To examine effects of season and territory size on DCX-ir cell density in the IPL and CL of the MC, ANOVA was performed using enclosure size and season with interactions on Minitab 16 Statistical Software. Tukey's post-hoc analyses were performed if main effects or interactions provided significant results. I predicted increased density of DCX-ir cells in both the IPL and CL of the MC in lizards from large enclosures as compared to those in the small. I also predicted increased densities in these regions in those lizards held during spring trials.

For analysis of the effect of T on neuroplasticity, separate regressions of all neuroplasticity variables against both T concentrations at the beginning and end of trials was run against all neuroplasticity variables, using Minitab 16 Statistical Software. To account for multiple comparisons, alpha level was corrected for multiple tests. As T levels varied in each season, regressions were performed for each season separately. I predicted that blood plasma T concentrations would have a positive relationship with brain volumes and the density of DCX-ir cells.

3 Results

3.1 Testosterone

Testosterone concentrations were elevated in the spring compared to the summer ($F=37.07$ $df=1, 17$ $p<0.001$; Figure 2) and elevated at the beginning compared to the end of each season ($F=62.638$, $df=1, 17$ $p<0.001$). Furthermore, there was a significant interaction between season and time ($F=28.618$, $df=1, 17$, $p<0.001$; Figure 2). In spring, there was a trend toward a reduction in T concentration from the beginning to the end of the season, but this was not statistically significant ($p=0.065$). In summer, however, T levels collected at the end of the season were significantly lower than those collected at time of capture ($p<0.001$). The T concentrations of lizards at their initial collection in spring and summer were not significantly different ($p=0.434$) but in the summer T levels after captivity had lowered from initial capture ($p<0.001$). There was not a significant

difference between the T concentration of wild and captive lizards in the month of June (p=0.140).

3.2 Behavior

There are no significant differences in the number of interacting lizards between spring and summer trials (χ^2=1.35, df=27 p=0.246). Similarly, there are no significant differences in the number of lizards observed between season (F=0.72, df=27, 1 *p*=0.403) or enclosure size (F=0.08, df=27, 1, p=0.779). There are no significant differences between the total number of displays between season (F=2.57, df=27, 1, p=0.122) or enclosure size (F=2.7, df=27, 1, p=0.113). However, the majority of aggressive responses occurred during the spring (Table 1). Furthermore, some individual behaviors not previously noted in the literature were observed. One male was observed inverting his hemipenes, excreting a foamy white secretion then retracting the hemipenes, followed by rubbing of the excretion onto the top of the cinderblock it was on. The same male was observed mounting an intruder male while biting his neck as he would during copulation with a female.

3.3 Cortical volumes

No difference was found in total MC volume between seasons (F=1.55, df=1, 19, p=0.229; Table 2, Figure 3) or enclosure size (F=0.50, df=1, 19, p=0.487), nor was there a significant interation between season and enclosure size (F=0.00, df=1, 19, p=0.973). Lizards from small enclosures had larger DC

volumes than lizards held in large enclosures (F=5.30, df=1, 17, p=0.036), while DC volume did not differ between seasons (F=1.20, df=1, 17, p=0.291) and no interaction was found (F=0.05, df=1, 17, p=0.833). No difference was found in the MC CL volume between season (F=1.28, df=1, 18, p=0.274), enclosure size (F=0.92, df=1, 18, p=0.351), or their interaction (F=0.98, df=1, 18, p=0.338). No effect of the random variable of enclosure location was found on either MC volume (F=2.78, df=7, 19, p=0.091), DC volume (F=1.62, df=6, 17, p=0.287), or MC CL volume (F=1.02, df=7, 18, p=0.508).

3.4 Immature neuron density

Lizards in large enclosures had higher density of DCX-ir cells in the MC CL than lizards from small enclosures (F=4.55, df=1, 25, p=0.036: Table 3, Figure 4). The density of DCX-ir cells in the MC CL was also greater in lizards from summer trials than spring (F=4.98, df=1, 25, p=0.044) but no interaction between experimental variables was found (F=0.50, df=1, 25, p=0.488). No difference in the density of MC IPL DCX-ir cells was found between enclosure size (F=1.93, df=1, 25, p=0.178) or season (F=2.34, df=1, 25, p=0.841). No effect of the random variable of enclosure location was found on MC CL DCX-ir cell density (F=1.05, df=13, 25, p=0.461) or MC IPL DCX-ir cell density (F=1.08, df=13, 25, p=0.444).

3.5 Testosterone and Neuroplasticity

Testosterone concentrations collected at the end of the summer trial negatively affected MC volume ($F=32.72$, $df=1, 7$, $p=0.001$, $r^2=0.82$, figure 5). Despite this significant relationship for the summer, no pattern was found for T concentrations collected at the end of spring trials and MC volume ($F=-3.02$, $df=1, 10$, $p=0.116$, $r^2=0.17$) despite a positive trend. No other relationships were found between T concentrations collected at the beginning of the spring or summer and all neuroplasticity variables ($p \geq 0.242$; Table 3).

4 Discussion

Here, I examined the territorial behavior and cortical plasticity of adult male *S. occidentalis* and tested the hypothesis that medial cortex (MC) or dorsal cortex (DC) plasticity is regulated by territory size and season, possibly through the action of T. I predicted that aggressive behavior and T levels would be high during the spring and low during the summer. Contrary to my prediction of higher behavioral responses during the spring, behavior (number of aggressive interactions, displays or total observations) did not vary between seasons. Therefore, no data supported the prediction of a seasonal difference in behavior. As expected, lizards had high levels of T during the spring and low levels by the end of summer. However, blood plasma T levels remained elevated at the beginning of summer trials indicating they had not yet dropped to non-breeding season levels at the start of summer behavioral trials. Overall, my predictions for

behavior were not upheld in this study and although T levels remained elevated at the beginning of summer, by the end they had dropped down to predicted baseline levels.

When looking at brain region markers of plasticity, I found dorsal cortex volume was greater in lizards from small enclosures compared to lizards held in large enclosures. This contradicts my prediction of greater brain region volumes in the larger enclosures. No differences between MC and MC CL volume were found based on the experimental variables of enclosure sizes and/or season. The MC data do not support my hypothesis while DC volume data suggest the opposite relationship than predicted. Neurogenesis, based on the density of DCX-immunoreactive cells, was predicted to be greater in the MC CL and MC IPL of lizards from spring and lizards placed in large enclosures. The density of DCX-ir cells in the MC CL was greater in lizards from large enclosures compared to those from small enclosures, a finding that supports my hypothesis. However, the density of DCX-ir cells in the MC CL was greater in lizards from the summer trial as compared to spring, which contradicts my hypothesis. No differences were found in the MC IPL between seasons or enclosure sizes. Testosterone concentrations were predicted to be positively correlated with cortical volumes and DCX-ir cell density during the spring and summer. I found T concentration levels at the end of summer trials were negatively related to MC CL DCX-ir cell density, which contradicts my prediction. Overall, several predictions were not upheld by my results but the relationships found do seem to indicate plasticity in

the MC and DC is in part regulated by the season and enclosure size. Data also indicate this may be mediated through the action of T.

4.1 Testosterone

Similar to previous studies (Mckinney & Marion, 1985; Davis, 1967), male *S. occidentalis* T levels were elevated during the spring months. As breeding season for *S. occidentalis* does often continue through mid-June and T levels can be elevated during this time of year (Mckinney & Marion, 1985; Klukowski & Nelson, 1998), the elevated levels at the beginning of summer most likely indicates I did not wait long enough for T levels to drop to post-breeding levels before beginning my summer trials. Based on these data, it is unclear how long lizards from our summer trials were exposed to the elevated T levels seen in samples collected when they were captured. Although T levels dropped significantly during the summer trial, the low levels seen at the end were expected based on previously studied patterns and was not likely due to negative effects of captivity (Mckinney & Marion, 1985; Sheldahl et al., 2000).

Despite being non-significant, my data indicates a trend for a drop in T levels during the spring period of captivity. Previous studies, using similar semi-natural enclosures, found that the hormonal profiles of female *S. virgatus* were not altered by long term confinement in a semi-natural enclosure (Weiss et al., 2009). However, previous experiments on *S. occidentalis* in our lab indicate that long-term captivity indoors can be detrimental to overall health (Personal observation). It is possible that, in the current experiment, captivity had a

detrimental effect on T levels even without the added stress of surgeries. It is also possible that the levels here dropped slightly due to the natural decline of T over the breeding season as seen in both *S. jarrovi* and *S. occidentalis* (Moore, 1987; Klukowski & Nelson, 1998). Also, there was a trend for testosterone concentrations in June to be lower in lizards that had been in captivity for seven weeks, compared to those wild-caught lizards for the beginning of the summer trials. This further supports the hypothesis that captivity negatively affects T levels but does not rule this out as a natural decline in T concentrations. Despite this possible effect of captivity, T concentrations did remain at elevated levels for the entirety of the spring trials and dropped to baseline levels by the end of summer trials. Further research should ensure T levels have dropped to non-breeding season levels before starting treatments during non-breeding season. The use of castration and T replacement would also be helpful in preventing this issue. Since surgeries are likely to cause increased stress, the effects of surgeries and captivity in semi-natural enclosures should be examined first. Despite the possible negative effects of captivity, these data follow seasonal profiles of free-living unmanipulated Eastern Fence Lizard (*Sceloporus undulatus)* males, a closely related species (Klukowski & Nelson, 1998). These data demonstrate that, like many populations of lizards, T levels change seasonally in the *S. occidentalis* in this study.

4.2 Behavior:

The main way in which these lizards communicate is through visual displays, which are most common during the breeding season, in territorial defense (Sheldahl et al., 2000; Fitch, 1940). Although clear and significant seasonal differences in fence lizard behavior have been described (Fitch, 1940; Klukowski & Nelson, 1998), the data collected in this experiment were insufficient to determine any differences in behavior occur in *S. occidentalis* between the seasons. This is partly due to the variability in display propensity during actual staged encounters for both the spring and summer. In addition, many of the lizards that did not display during behavioral trials were seen exhibiting push-up displays outside of recorded trials (personal observation). As data were collected only on focal lizards in each trial, other lizards seen exhibiting aggressive behaviors did not get included if they were not the focal lizard. Further studies in this manner should video record all activities of the lizards to be able to collect more data.

However, it is possible that this population of *S. occidentalis* does not have seasonal differences in aggressive behaviors. This could occur if T is not a main factor stimulating aggression in these lizards. Yarrow's Spiny Lizard (*Sceloporus jarrovi*) is characterized as not being territorial out of the spring breeding season, but some male *S. jarrovi* continue displaying during the summer (Moore & Marler, 1987; Marler & Moore, 1989). Also, Moore (1987) found that even after castration, some male *S. jarrovi* continued to perform aggressive displays. Testosterone may have a prolonged activational effect on

these behaviors and territorial abilities of *S. jarrovi*, allowing the elevated levels during spring to mediate behaviors long after the T levels have lowered. Since *S. occidentalis* do show at least some territorial behavior year round, variation between seasons may not be as pronounced in *S. occidentalis* as it is in *S. jarrovi*.

Despite no significant differences between seasons, descriptive statistics on behavioral data indicate several trends. With 81% of the total trials positive for an aggressive interactions seen occurring in the spring, it seems unlikely that behavior between seasons is the same. This is further supported with 92% of the total aggressive displays within trials also occurring during the spring. Also worth noting is the difference in the number of lizards who were noted as having an aggressive interaction to intruders between spring and summer. During the spring trials 64% of the total lizards were recorded having at least one aggressive interaction while this percent dropped to 36% during the summer. These are supported by data from other populations of *S. occidentalis*, where aggressive responses are significantly increased during the spring season and drop drastically by early June (Klukowski & Nelson, 1998). Additionally, in previous studies on this population of *S. occidentalis* there was no difference in behavior between T treated and castrated lizards, indicating T concentrations alone may not cause behavioral changes in this population (Pollock et al., 2012). It may be that seasonal changes in behavior are not present in this *S. occidentalis* population but despite this possibility, it seems more likely that the collected data was insufficient to detect a seasonal difference. By gathering more data on the

behavior of lizards during the various seasons and through the use of video recording devices, a difference may be determined, if it truly exists.

In this study, lizards in both sizes of enclosures displayed and reacted aggressively. Previous studies on these lizards in semi-natural enclosures have found that they will continue to display their normal aggressive behaviors (Tarr, 1977, Sheldahl et al., 2000). Males from both size enclosures displayed aggressive territorial responses through-out the trials. Therefore, male *S. occidentalis* can be coerced into defending a “territory” in a semi-natural enclosure and this technique can be helpful in future studies manipulating or controlling for territory size.

4.3 Neuroplasticity and enclosure size

I found the DC to be larger in lizards from smaller enclosures, a result that contradicts my prediction of increased volumes in larger enclosures. As the DC has been tied to both spatial and non-spatial tasks (Font et al., 1991), it is possible that differences in the demand for non-spatial tasks, such as social interaction, may drive regional differences here. In Mountain Chickadees (*Poecile gambeli*) it was found that the hippocampus of dominant chickadees had more neurogenesis than paired subordinates (Pravosudov & Omanska, 2005), suggesting cues of social dominance may play a role in hippocampal plasticity. In an experiment examining the MC and DC of conspecific lizards with differing breeding strategies, lizards that did not hold a territory and had very little interaction with females had smaller DC volumes than territorial consepecifics

(LaDage et al., 2009). Perhaps, like the hippocampus of *P. gambeli*, the volume of the lizard DC is regulated partly through social interactions. The lizards in smaller enclosures were more likely to have additional interactions with the resident female because these enclosures were limited in space compared to the large enclosures. In *S. occidentalis*, male-female interactions are of vital importance when determining a territory. Because males defend a territory partly to ensure reproductive success (Fitch, 1940), it is obvious that male-female interactions are important events and these events may also be important for brain plasticity. Territories are chosen based on their overlap with the home-ranges of females (Sinervo et al., 2000), so spatial memory formation may be based on social interaction and their effect on cortical volumes may depend on male-female interactions. To examine this, future studies may want to vary female number in the different enclosure sizes to examine the effect of opposite-sex conspecific interactions on cortical neuroplasticity.

While DC volume changes opposed my prediction, I did find that MC CL neurogenesis was increased in those lizards held in large enclosures. In the wild, lizards live in complex spatial environments that include basking sites, food or water sources, and interactions with other animals, including conspecifics, prey, or predators. Diversity in the experiences of these complex situations by separate individuals would therefore represent a variation in their spatial demands. I predicted variability in spatial demands will be recapitulated in the variability of MC and DC neuroplasticity. The stimulatory effect of navigating the more enriched large enclosure may be driving this increase in neurogenesis. The

hippocampus of mice undergoes increased neurogenesis when they are placed in enriched environments (Kempermann et al., 2002). Many factors such as stress reduction, increased enclosure size, and increased exercise have been posited to cause the neurogenic effect in animals exposed to enriched environments (Klempin & Kempermann, 1997; Kempermann et al., 2002; Fares & Belmeguenai, 2013; Tanti et al., 2013; Birch et al., 2013). Free-living lizards may also have individual variation in environmental enrichment. Larger MC volumes are found in those lizards that are actively foraging versus lizards sitting-and-waiting for prey (Day et al., 1999). Lizards that are actively seeking prey, therefore, are challenged with more novel environments as they experience their spatial world via foraging. A *S. occidentalis* living in the wild would have these same opportunities. A lizard in captivity (especially in an indoor, non-semi-natural enclosure) loses these demands on spatial abilities, and much like a sit-and-wait predator, will not be as challenged. Indeed, captivity in small simple cages has a negative relationship with cortical volumes (Pfau et al., 2010). The reduction in MC neurogenesis seen in small enclosures is may be due to a lack or decrease in the spatial complexity necessary to induce cortical growth. Current research shows that increasing spatial complexity can have positive effects on neurogenesis beyond those of stress reduction (Fares & Belmeguenai, 2013; Tanti et al., 2013) and increases in activity (Birch et al., 2013). Therefore increases in MC neurogenesis in lizards from large enclosures are likely due to the increased spatial demands associated with the increased enclosure size. Further research may examine the effects of enclosure size on progenitor cells

and neurons of the lizard cortex to determine exactly how increased spatial demands can lead to increases in neurogenesis.

The increase in MC neurogenesis and a decrease in DC volume for lizards held in large enclosures may represent a trade-off in neuroplasticity between the MC and DC. This trade-off between spatial regions is also observable in studies on the human hippocampus. The posterior portion of the hippocampus and years spent as a London taxi driver are positively related, whereas this relationship is negative in the anterior portion. When looking at bus driver controls, no such size dimorphism was indicated for either region (Ghaem et al., 1997). Taxi cab drivers also performed better on tests of familiar spatial problems but performed poorly in tests of novel spatial ability when compared to bus drivers (Maguire et al., 2006). The growth in the posterior hippocampus and its positive relationship with scores of spatial abilities in familiar environments indicates this region may be involved with the formation of a cognitive map. Lesions to the MC of lizards and turtles cause them to lose the ability to spatially navigate in a recently learned spatial environment, indicating MC lesions result in the loss of recently acquired spatial memories (Day et al., 2001; López et al., 2003). The MC in lizards may therefore be functionally similar to the posterior hippocampus in humans and could be involved with the formation of a cognitive map. Neurogenesis within the cell layer of the MC is increased in those lizards placed into larger enclosures while there was no concurrent increase in MC CL volume or MC volume. This indicates a change in the total number of cells is not likely and instead there is probably a difference in the number of new neurons

being integrated. Furthermore, the decrease in DC volume seen in lizards from larger enclosures may be similar to the decrease seen in the anterior hippocampus of taxi cab drivers. This loss in DC volume may be a cost associated with increased MC neurogenesis. Lesions to the DC do not impair spatial abilities in a learned environment as much as those to the MC (Day et al., 2001) and, although still related to spatial memory, may be more important for the processing of novel spatial stimuli just like the human anterior hippocampus. Taken together, these studies and my results support the hypothesis that the MC is involved in the formation of a cognitive map and that neurogenesis may be important for forming that map. Furthermore, there may be a tradeoff between parts of the brain responsible for creating cognitive maps of the environment (the MC and posterior hippocampus) and those responsible for navigating in novel environments or for navigating based on other factors such as social interactions (the DC and anterior hippocampus). The ability to navigate novel and learned spatial environments can be tested in lizards previously held in differing sized enclosures to determine and functional gain or loss associated with MC and DC plasticity.

Alternatively, the increased neurogenesis in lizards from larger enclosures may also be due to differences in the environment experienced by the lizards in these enclosures. Basking time, enclosure temperature, and time of access to shade and sunlight may all differ based on enclosure size. In this way, simple increases in stimulation by spatial navigation may not be the only variable responsible for the increased MC neurogenesis. In the Tenerife Lizard (*Gallotia*

galloti), decreases in temperature and light exposure impede the reactive neurogenesis in response to cortical lesions (Ramirez et al., 1997). Also, neurogenesis is highest during times of the year with increased temperature and increased daylight hours as the seasons change (Delgado-Gonzalez et al., 2007). Given that the larger enclosures had more basking sites, less shade, and likely more access to sunlight, the difference seen between treatment groups may be a result of a difference in temperature and exposure to direct sunlight. In the wild, the lizards that have the larger territories or higher quality territories may have the most preferable basking sites and therefore be able to optimally regulate their temperature. Basking sites have been implicated as one of the resources that initiate a lizard's defense of a territory (Sheldahl et al., 2000). Behavioral data here indicate no difference in how often lizards were visible to researchers in the large enclosures versus the small enclosures, indicating no difference in activity. Despite this, the differences between enclosure temperature and amount of time with access to direct sunlight may be enough to drive a difference in the neuroplasticity of the MC. A study done in the lab may be able to control environmental factors such as individual enclosure temperatures and sunlight access, which is more difficult or impossible in a study such as this.

4.4 Seasonal variation in neuroplasticity

I determined that the number of new neurons incorporated into the MC CL was lower during the spring. During the spring study, there were four instances of rain storms that likely prevented basking and drastically lowered the

environmental temperature, sometimes for several days. In European Wall Lizards (*Podacris hispanica*), harsh, winter-like conditions prevent the reactive neurogenesis that leads to new MC growth (Molowny et al., 1995) and have been shown to decrease migration speed of immature neurons traveling to the MC (Ramirez et al., 1997). Although, in those experiments, the lizards were exposed to chronic inclement conditions, it may be possible that the acute periods of lowered temperature and light seen in the spring trials of this study may be detrimental to neurogenesis as well. Further research may examine the effect of both chronic and acute variations in environmental variable such as direct sunlight and temperature to determine what effects they may individually have on cortical neuroplasticity in *S. occidentalis*.

This increase in new neuron number in the MC CL during the summer may also indicate that the summer season has a stimulatory effect on neurogenesis. Seasonal variations in neuroplasticity have been established both in the song control regions of songbirds and several regions of the lizard forebrain (Nottebohm, 2002; Delgado-Gonzalez et al., 2008). In previous studies, lizards had a significant increase in total brain neurogenesis during spring and summer. Also, in the wild, a larger amount of neurogenesis occurred during the spring (Ramirez et al., 1997; Delgado-González et al., 2007). In the lab, studies have indicated the individual importance of photoperiod and temperature on cortical neuroplasticity, low levels of both appear to attenuate cortical neuroplasticity (Ramirez et al., 1997). It seems possible that the increased temperatures and day length during the summer would drive neuron number

even higher during the summer as compared to the spring. Several spring storms may also have been detrimental to the neurogenesis and migration of newly born cells during the breeding season.

4.5 Neuroplasticity and androgens

In songbirds, seasonal differences in neuroplasticity are related to changes in natural photoperiod and gonadal hormones (Nottebohm, 2002). In some species these brain regions are also responsive to changes in photoperiod absent from changes in T (Nottebohm, 2002; Strand & Deviche, 2007; Strand et al., 2009; Ball & Balthazart, 2010). Still, T directly affects these song control regions, independent from photoperiod, to modulate plasticity and induce singing behaviors (Dloniak & Deviche, 2001). Despite these connections between T and songbird neuroplasticity, no such relationship has been described in a reptile. Here, I found that MC volume is negatively associated with T levels during the summer. Since T levels were so drastically low at this point, the degree of variation in T levels seen here may not be biologically relevant. No such relationship appears in the spring, in fact, the relationship between T and MC volume during the spring has a positive trend.

This seasonal difference in the effects of T is not surprising. Elevated T levels can activate sexual behaviors that enhance male fitness during the breeding season (Wingfield et al., 1990; Sinervo et al., 2000). Despite this seasonal benefit T provides for male *S. occidentali*s, it may also have several detrimental effects. Prolonged elevation of T has been experimentally tied to

decreased survivability (Dufty, 1989), increases in predation probability (Marler and Moore, 1989), and immune function inhibition (Hillgarth and Wingfield, 1997). In *S. occidentalis* higher T levels can lead to higher levels of parasite load (Pollock et al., 2012). Therefore, increased androgens year round would be costly, so many vertebrate species save the powerful effects of T for only part of the year, such as a breeding season (Soma et al., 2008). Testosterone levels here were at their lowest at the end of summer trials, well out of the breeding season

It has been shown in the Green Anole (*Anolis carolinensis*) that the cells in the MC express mRNA for androgen receptors, indicating androgen signals may be received in the MC (Rosen et al., 2002). Androgens have been shown to enhance the survival of neurons in the hippocampus of male rats (Spritzer & Galea, 2007) and in the songbird HVC (Rasika et al., 1994). Here, we saw an increase in the amount of neurogenesis during the summer, a period of the year marked by lower T levels. Kirn et al. (1997) observed that peaks in the number of new neurons being added to the HVC of the canary brain occurred just a month after a peak was seen in the number of pyknotic neurons. This suggests a possible connection between the vacancies left by dying neurons and the replacement of those through neurogenesis. Therefore neuroprotection by T would increase cell lifespan, decreasing neurogenesis by lowering the number of vacancies left by apoptotic cells. In the present study, I saw an increase in the density of immature neurons during the summer when T was low. Neurons in the MC may follow a similar pattern of survival, death, and replacement as seen in

the songbird song control nuclei (Nottebohm, 2002; Kirn et al., 1997). In this way, the low levels of T seen during the summer may be unable to protect MC CL neurons from going through apoptosis, leading to increased numbers of new cells as they are integrated to replace those lost. Future studies should quantify apoptotic cells to determine the amount of cell death occurring within these regions. This would make it possible to examine the effect of T on the number of cells going through apoptosis and determine if it is a factor leading to neuroprotection.

The change from a positive relationship in spring to a negative one in summer indicates that seasonal differences may exist in the way T affects neuron survival and neurogenesis. Seasonal variation in T receptor regulation is present in other animals as well. In Siberian hamsters, another seasonally varying hormone, melatonin, is known to modulate sensitivity to T (Demas et al., 2004). In the songbird HVC, higher levels of androgen receptor mRNA are found during the breeding season, indicating changes in T receptivity throughout the year (Soma et al., 2008). In lizards, it is unknown if AR expression varies seasonally but based on previous data and results from this study, *S. occidentalis* T levels do change throughout the year in this population (Taylor, unpublished data). Although AR itself has not been examined in lizards, differences in the production of cofactors regulating T aromatization have been found between seasons in *A. carolinensis* (Cohen et al., 2011). Thus, variations in androgen receptivity, hormone cofactors, and modulation by other seasonally relevant hormones may regulate how T affects neuroplasticity. A breeding

season environment may drive changes to the male *S. occidentalis* MC so that T acts in a neuroprotective way, increasing survival of neurons and enhancing spatial cognition. During times of low T the neuroprotection may be lost, causing neurons to die leading to the incorporation of new cells in the MC CL seen during the summer. Further studies into the lizard cortex should examine T cofactors, androgen receptors, and other hormonal interactions to determine if they differ based on season.

Conclusions

Here, I have shown several novel behaviors not previously documented in this species, a male-male sexual interaction and a possible new way for chemically marking territories. No seasonal difference in aggressive behaviors was detected in these lizards. Detection of significant differences in behavior between seasons may require a much larger sample size or increased observation time, but descriptive statistics from my study indicate a difference is likely to be found. Variations in neuroplasticity based on territory size, such as increases in the density of new neurons in the MC in those lizards from large enclosures, indicate demands placed on spatial behavior are most likely driving increases in neuroplasticity. Furthermore, the DC may be involved in different functions rather than simply spatial memory. Possibly, the separate regions involved with spatial memory are involved with more specific tasks, such as learning novel environments vs. navigating familiar ones, and there may be costs

to one region when the other is enhanced. Finally, the differential effect of T on MC volume based on season found in *S. occidentalis* is the first such finding in a reptile to my knowledge.

The further study of the *S. occidentalis* male cortex and the use of this system as a model for studying neuroplasticity are recommended for several reasons. First, the phenomenal regeneration abilities of the reptile cortex make it a prime candidate for research on possible treatments for brain damage and neurodegeneration. Second, these data support the hypothesis of homology between the reptile cortex and human hippocampus through experience-based plasticity, and possibly through a trade-off between MC and DC plasticity. Finally, besides the avian song control nuclei, this is the first model of neuroplasticity to indicate an effect of season on androgen modulation of neuroplasticity. Further research on this model organism could provide answers to many specific questions on seasonal variations in behavior and brain plasticity. Our understanding of neuroplasticity, and in particular neurogenesis, would benefit through future analysis of the experience based changes and seasonal variation on neuroplasticity seen here.

Table 1: Descriptive statistics of behavioral data from both spring and summer trials. All interactions indicated by this table are based on resident male aggressive responses to intruder males. The sum of summer and spring values were used to determine what percentage of total behaviors occurred within each season.

	Spring	Summer
Number of observations	118	139
Number of interactions	29	7
% observations with an interaction	25%	5%
% total number interactions	81%	19%
Total Number of push-ups	95	55
% total push-ups	63%	37%
Total number of aggressive displays	593	55
% Total number of aggressive displays	92%	8%
Number of lizards that interacted	7	4
% Lizards interacting	50%	29%
% Interacting lizards	64%	36%

Table 2: MANOVA results of the main effects of season and enclosure size and season effect on neuroplasticity variables. Statistically significant effects ($p<0.05$) are indicated by bold italics.

	Season			Enclosure size		
	F	df	p	F	df	p
Medial Cortex Volume	1.55	19,1	0.229	0.5	19,1	0.487
Dorsal Cortex Volume	1.2	17,1	0.291	***5.3***	***17,1***	***0.036***
Medial Cortex Cell Layer volume	1.28	18,1	0.274	0.92	18,1	0.351
Medial Cortex Cell layer DCX-ir cell density	***4.55***	***25,1***	***0.044***	***2.08***	***25,1***	***0.036***
Medial Cortex Inner plexiform layer DCX-ir cell density	2.51	25,1	0.127	1.93	25,1	0.178

Table 3: Results from the regression analysis to determine the effect of testosterone concentrations (at the beginning and end of each season) on neuroplasticity variables. Statistically significant relationships ($p<0.05$) are indicated by bold italics.

		T at the beginning of the season				**T at the end of the season**			
		t	df	r^2	p	t	df	r^2	p
Spring	Medial Cortex Volume	-0.88	9	-2.59	0.405	1.74	10	16.8	0.116
	Dorsal Cortex Volume	-0.58	9	-7.96	0.578	1.388	10	8.47	0.199
	Medial Cortex Cell Layer volume	-0.637	9	-7.05	0.541	0.447	10	-8.7	0.666
	Medial Cortex Cell layer DCX-ir cell density	0.64	10	0	0.537	-1.06	12	1.04	0.311
	Medial Cortex Inner plexiform layer DCX-ir cell density	0.915	10	-1.66	0.384	-1.022	12	0.38	0.328
Summer	Medial Cortex Volume	-0.259	6	-18.41	0.806	***-5.72***	***7***	***81.93***	***0.001***
	Dorsal Cortex Volume	0.245	4	-30.72	0.822	-0.141	5	-24.38	0.895
	Medial Cortex Cell Layer volume	1.37	5	14.95	0.242	-0.479	6	-14.74	0.652
	Medial Cortex Cell layer DCX-ir cell density	-0.499	7	-12.02	0.636	-0.393	11	-8.33	0.703
	Medial Cortex Inner plexiform layer DCX-ir cell density	0.233	7	-15.61	0.822	-1.291	11	5.7	0.226

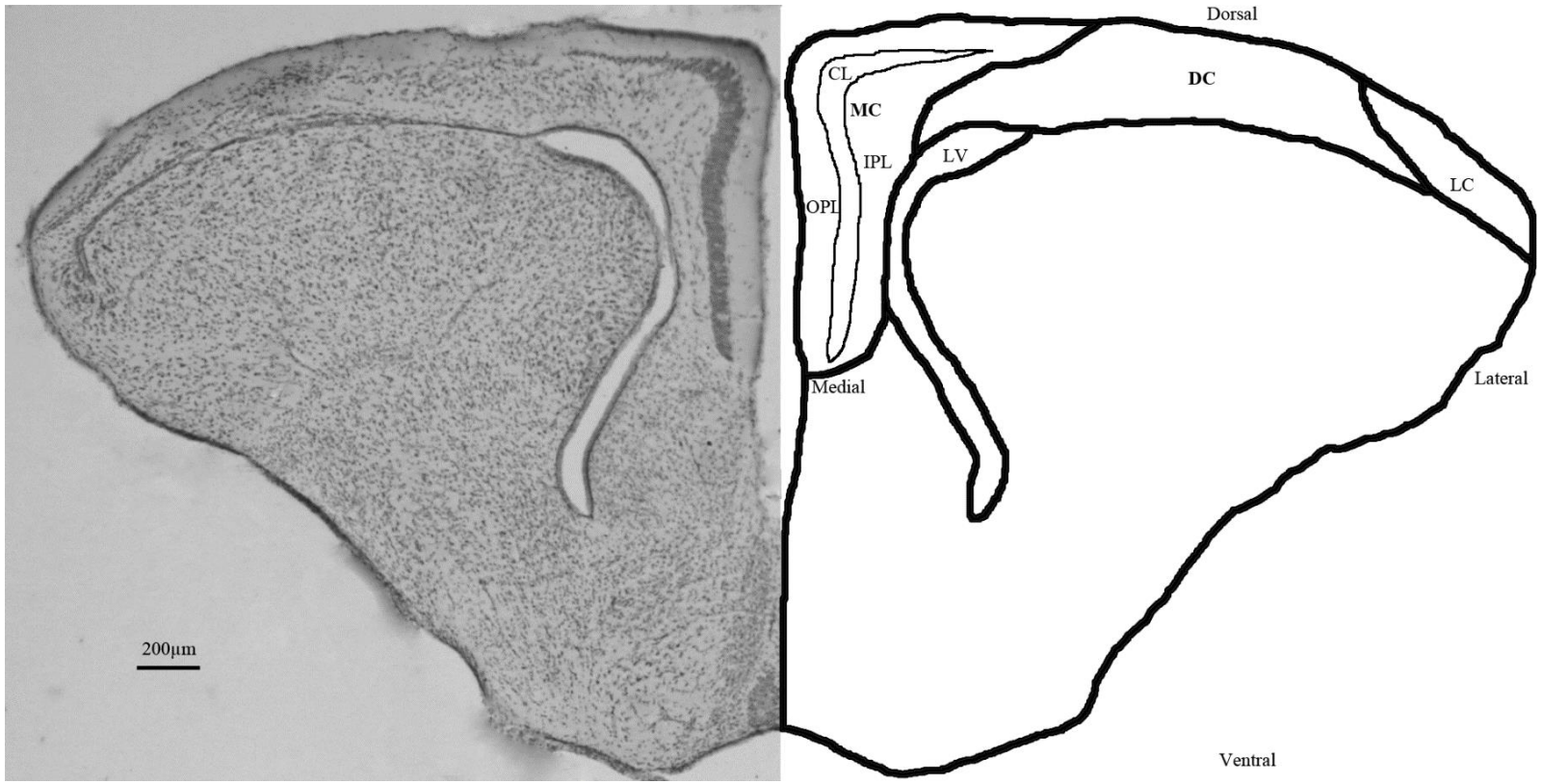

Figure 1: Cresyl Violet stained section through the telencephalon of an adult male *Sceloporus occidentalis*. medial cortex, MC; dorsal cortex, DC; lateral cortex, LC; cell layer, CL; inner plexiform layer, IPL; outer plexiform layer, OPL; lateral ventricle, LV.

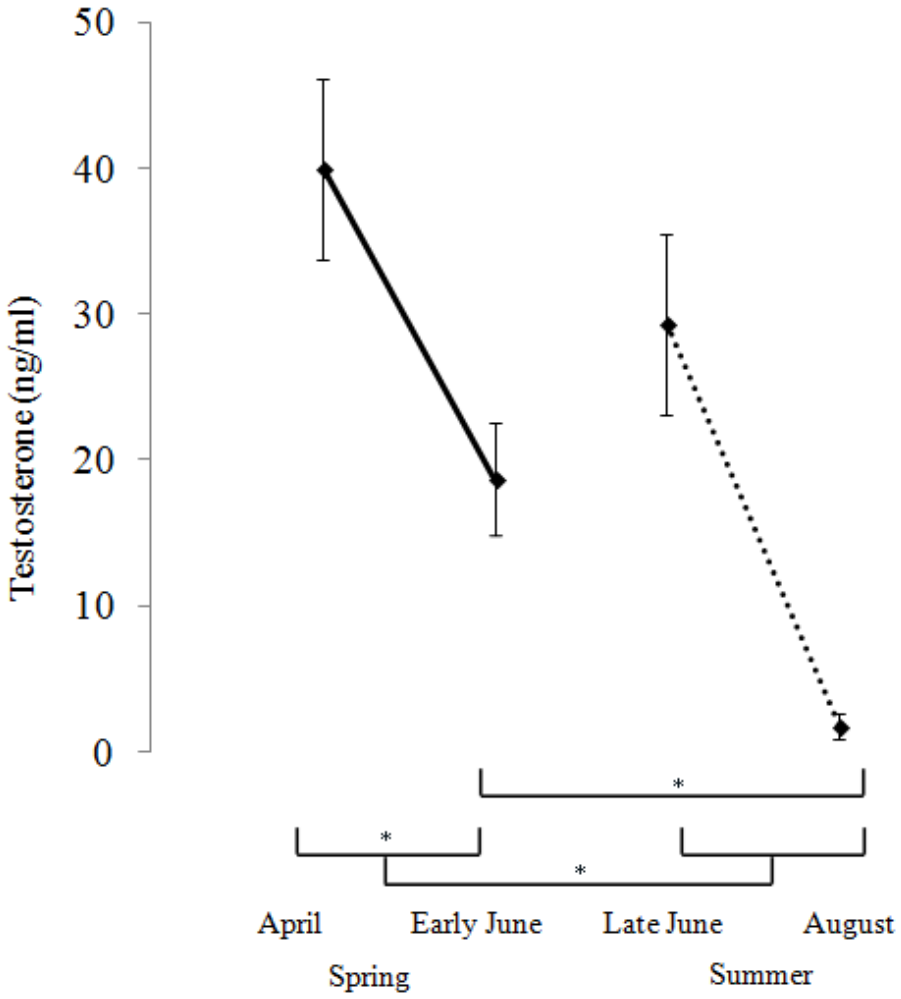

Figure 2: Mean testosterone concentrations (ng/ml; +/- SEM) in male *Sceloporus occidentalis*. Lizards were caught in April and sacrificed in early June (solid line, n=11) and caught in late June and sacrificed in August (dotted line, n=8).

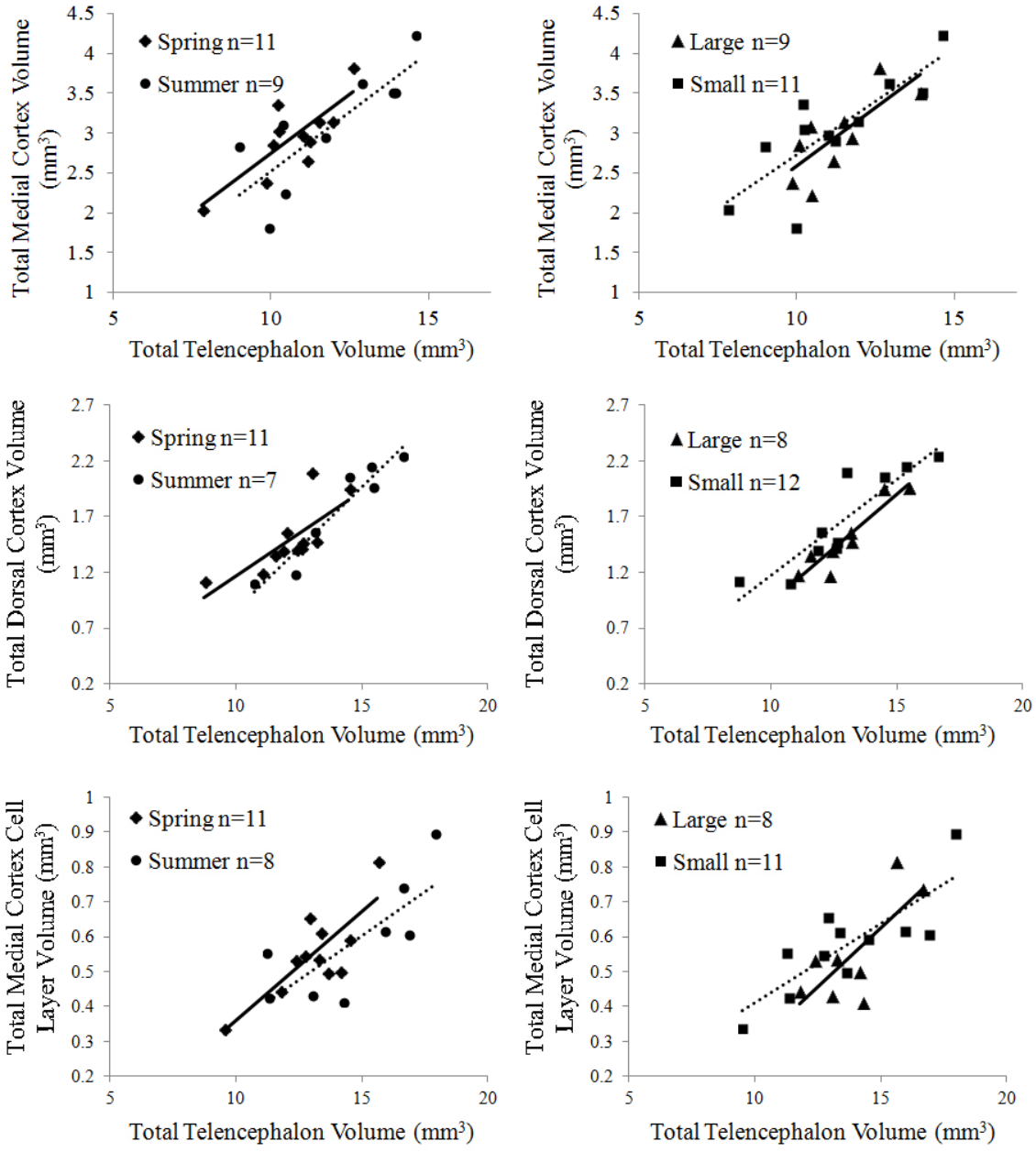

Figure 3: The volume of each brain region (MC, DC, and MC cell layer) for each lizard in relation to adjusted total telencephalon volumes (TT minus respective cortex volume) comparing between seasons and enclosure size. Males held in small enclosures had larger dorsal cortex volumes than those in large enclosures ($p=0.036$).

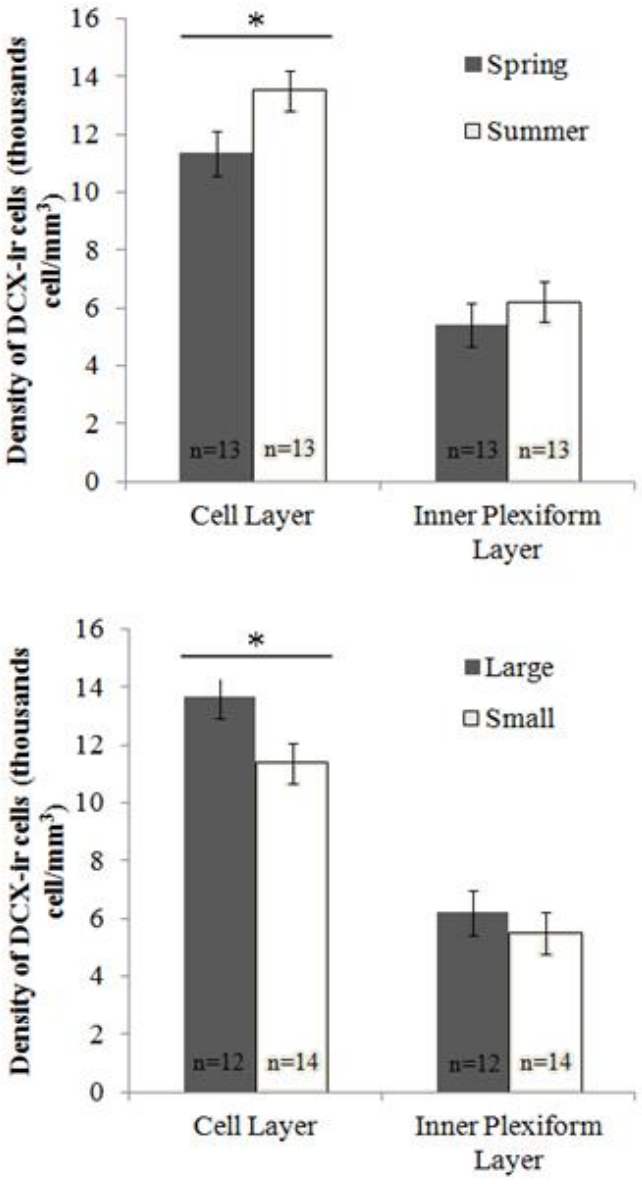

Figure 4: Mean density of doublecortin-immunoreactive (DCX-ir) cells (thousand cells/mm^3; +/-SEM) in the cell layer (CL) and inner plexiform layer of the medial cortex in male *Sceloporus occidentalis* from the spring or summer (Top panel) or housed in small or large enclosures (Bottom panel). Significant differences ($p<0.04$) are indicated by an asterisks.

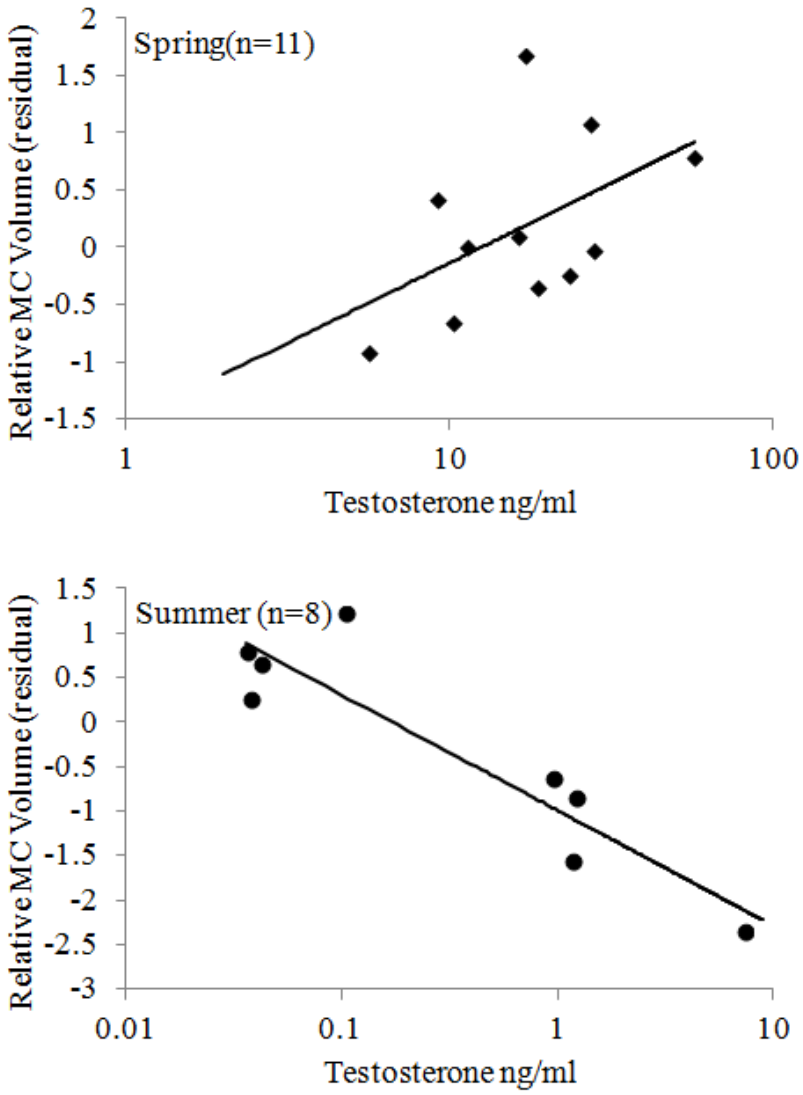

Figure 5: Regression of the relative medial cortex volume (Residuals of the regression of MC and total telencephalon) and log-transformed testosterone concentration (ng/ml) collected at the end of treatment in spring and summer.

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