Interaction Between Territoriality, Spatial Environment, and Hippocampal Neurogenesis in Male Side-Blotched Lizards

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Differences in an animal’s spatial environment can have dramatic effects on the brain, especially the hippocampus, the area of the brain heavily involved in spatial processing. Animals in spatially impoverished environments have decreased hippocampal attributes. However, we do not know if differences in the spatial environment differentially interact with territorial status, which also covaries with hippocampal attributes. Here, we asked whether territoriality and differential spatial-area use interact to generate different effects on cortical attributes (reptilian hippocampal homologue) in lizards. We compared medial and dorsal cortical attributes between territorial and nonterritorial morphotypes of side-blotched lizards, Uta stansburiana, in larger versus smaller (i.e., spatially impoverished) enclosures. We found that territorial males had increased neurogenesis rates in their medial cortices in larger enclosures when compared with their siblings in smaller enclosures; nonterritorial males had low levels of neurogenesis regardless of enclosure size. Enclosure size had no significant effect on cortical volumes or the total number of neurons in either cortical region. These results suggest that territorial morphotypes may be more sensitive to changes in the spatial environment, thus leading to increases in regulation of neurogenesis in the face of increased spatial processing and physical activity demands.

Keywords: environmental enrichment, lizards, neurogenesis, spatial processing, Uta stansburiana

Changes in an animal’s spatial environment can have dramatic effects on the brain, especially the hippocampus, the area of the brain heavily involved in spatial processing (Van Praag, Kempermann, & Gage, 2000). Typically, alterations to an animal’s spatial environment are characterized by increased spatial area and spatial complexity. Both of these factors have been shown to induce morphological and physiological changes in the brain, including increased brain size, neurogenesis, gliogenesis, and synaptogenesis (e.g., Kozorovitskiy et al., 2005; Rampon et al., 2000; Van Praag et al., 2000).

The relationship between increased spatial-area use and complexity and changes in the brain are presumed to be mediated through both an arousal response and increased involvement of spatial learning and memory (sensu; Van Praag et al., 2000). The arousal response occurs when the opportunity for physical activity increases due to increased spatial-area use. Voluntary physical movement alone has been shown to be sufficient to produce an increase in cell proliferation rates in the dentate gyrus of rodents (Van Praag, Christie, Sejnowski, & Gage, 1999a; Van Praag, Kempermann, & Gage, 1999b). Increased spatial-area use and complexity also increase the demands on spatial learning and memory. Navigation in a more spatially complex environment increases demands on spatial processing abilities. Similar to the arousal response, increased demands on spatial learning and memory have also been shown to increase various attributes of the hippocampus, including adult neurogenesis rates, volume, and neuron number (Ambrogini et al., 2000; Döbrössy, Auroussou, Le Moal, Piazza, & Abrous, 2003; Gould, Boylin, Tanapa, Reeves, & Shors, 1999; Hairston et al., 2005; LaDage, Roth, Fox, & Pravosudov, 2009; LaDage, Roth, Fox, & Pravosudov, 2010). Although teasing apart the relative contributions of the arousal response and spatial learning and memory use has proven difficult, a combination of increased physical activity as well as increased spatial learning and memory use appear to occur when an animal moves from an area of lesser to greater spatial size and complexity (Kempermann, Kuhn, & Gage, 1997).

What is unclear, however, is whether specific life-history traits associated with differential demands on spatial processing in an...
ecological context affect how individuals respond to variables shown to be important in modulating hippocampal changes (e.g., alterations in spatial-area use or spatial complexity). For example, individuals of a particular species may differ in their territorial behavior; some animals may defend clearly delineated territories, and others may exhibit nonterritorial behavior. Territorial animals maintain territory boundaries through competitive exclusion. Thus, we may expect that these animals are more highly reliant on spatial learning and memory so as to remember and maintain territorial boundaries, as well as determine, spatially, where their territory-holding neighbors are located (Falls, 1982; Godard, 1991; McGregor & Westby, 1992; Sherry, 1998). Further, territorial males may engage in increased physical activity during territorial defense. Nonterritorial animals, however, do not maintain territories and thus are expected to be less reliant on spatial learning, spatial memory, and physical activity. Because of this disparity in the expected demands on spatial processing and physical activity between territorial and nonterritorial animals, we may expect to see those differences reflected in their responses to alteration in spatial area.

We tested the idea that an increase in spatial-area use can lead to differential effects on individuals, contingent upon territorial status. We used male side-blotched lizards (Uta stansburiana), which have been shown to have alternative spatial-use strategies that correlate with the three genetically distinct morphotypes found in this species (e.g., Sinervo, Bley, & Adamopoulou, 2001). Orange and blue males hold territories; yellow males are sneakers who do not defend territories (Sinervo & Lively, 1996; Sinervo & Zamudio, 2001). The orange morph occupies and defends large territories (~40 m²), the blue morph occupies and defends smaller territories (~23 m²), and the yellow morph does not hold or defend a territory and its home range is smaller than the territories of the other two morphs (~20 m²) (Calsbeek & Sinervo, 2002; Sinervo & Lively, 1996; Zamudio & Sinervo, 2000). As with other territorial species, territory patrol and defense likely cause an increased demand on spatial use and memory (Sherry, 1998), as well as physical activity. Indeed, brain attributes in this species also differ based on territorial status—territorial males (orange and blue morphotypes) have larger dorsal cortical volumes than nonterritorial males (LaDage, Riggs et al., 2009). Thus, in this species, there appears to be a relationship between territorial status and the underlying neural mechanisms largely responsible for spatial processing.

We assume that territorial males should rely more heavily on spatial processing and increased physical activity during territorial defense and we asked whether they are more sensitive to changes in the spatial environment than their nonterritorial counterparts. First, we tested whether an increase in spatial area can induce differences in cortical attributes such as volume, total neuron number, and neurogenesis in the side-blotched lizard. We assumed that an increase in spatial-area use would be reflected by an increase in cortical volume, neuron numbers, and neurogenesis rates in both territorial and nonterritorial males housed in larger spatial enclosures. Second, we asked if differences in territorial status could differentially interact with increased spatial-area use. We predicted that spatial processing and physical activity might be more important for territorial than nonterritorial males, thus territorial males should be more sensitive to alterations in spatial-area use. Therefore, we predicted that in larger enclosures, territorial males would have an additional increase in cortical morphology and neurogenesis when compared with nonterritorial males in a similar-sized enclosure.

Method

Subjects and Housing Conditions

Male and female adult side-blotched lizards were collected from the field near the Los Baños Grandes area in California, and transported to the University of Nevada, Reno laboratory in March, 2010. These individuals were assigned to breeding enclosures based on morphotype. Resultant offspring were therefore primarily homozygous for morphotype (orange, blue, or yellow). Of the offspring produced from the breeding colony, eight pairs of full sibling brothers (N = 16 animals, total) were used in this experiment; four pairs were territorial (orange or blue) and four were nonterritorial (yellow). Because our previous study indicated no neurobiological difference in cortical volume between the orange and blue territorial males (LaDage, Riggs et al., 2009), our current analyses were based on territorial status rather than morphotype. Thus, we compared territorial males (orange and blue) with nonterritorial males (yellow). All animals were fed live crickets dusted with calcium and vitamins and supplied with water ad libitum. The temperature of the laboratory was maintained at 20 °C. Cages were supplemented with above-cage basking lights, which provided a thermal gradient from 25 °C to 40 °C. All lights were kept on a cycle of 14L:8D.

Experimental Design

After hatching, male offspring were individually housed (enclosure size: 22 × 14 × 13.5 cm) for three months. After three months, each brother dyad was randomly assigned to one of two treatment groups—a larger or a smaller enclosure. The larger enclosures measured 144 × 53 × 38 cm and the smaller enclosures measured 22 × 14 × 13.5 cm; this represented a 25-fold difference in spatial area. All enclosures were filled with sand, basking rocks, and a basking light. Each lizard was also filmed over 8 hr, consecutively, on a random day during the treatment to monitor movement and spatial use of his enclosure; all videotaping was made from lights on (8:00 a.m.) until 8 hr after lights on (4:00 p.m.). Individuals were not videotaped at night, as heat sources are not available during the night, thus, movement was physiologically and behaviorally restricted, compared with that seen during daylight hours (personal observation). Using a grid overlay on the video footage, we were able to score distance traveled within an individual’s enclosure. All animals were kept in their treatment groups for 5 months. The last month before sacrifice encompassed the time frame in which all males were at breeding age, exhibited increased levels of testosterone, and established territories in their natural habitat (e.g., Wilson & Wingfield, 1994; Ferguson & Fox, 1984). At this age, the snout-vent length of males can range, on average, from 4.2–5.3 cm (Nussbaum & Diller, 1967).

Brain Extraction and Staining

At the end of the experiment, the 16 males were sacrificed, following the procedure of LaDage, Riggs et al. (2009). In short,
the lizards were anesthetized using a lethal overdose of Nembutal (0.05 ml of 50 mg/ml Nembutal). The lizards were then transcardially perfused with 0.1 mole phosphate-buffered saline for 10 min, followed by a 15–20 min perfusion of buffered 10% methanol-free formalin (from paraformaldehyde). Brains were then extracted and postfixed for 24 hr. Brains were cryoprotected in 15% sucrose for 1 day, 30% sucrose for an additional day, then flash frozen on dry ice and stored at −80 °C. Brains were sectioned on a cryostat (−20 °C; Leica CM 3050S, Nussloch, Germany) in the coronal plane every 40 μm. Every third section was mounted and Nissl-stained with thionin (see Figure 1a). A second set was processed for doublecortin immunohistochemistry (see Figure 1b).

Immunohistochemistry

To visualize neurogenesis, brain sections were processed for doublecortin, an endogenous protein expressed by immature, migrating neurons (Balthazart, Boseret, Konkle, Hurley, & Ball, 2008; Brown et al., 2003; Couillard-Despres et al., 2005; Delgado-Gonzalez, Gonzalez-Granero, Trujillo-Trujillo, García-Verdugo, & Damas-Hernandez, 2011; Hairston et al., 2005; Luzzati, Bonfanti, Fasolo, & Peretto, 2009; Rao & Shetty, 2004). Doublecortin labeling thus only provides a snapshot of the number of immature, migrating neurons. Eventually, these migrating neurons would have matured and integrated into the existing neural network. The production of new neurons in lizards is similar to that seen in the mammalian hippocampus, in that new neurons are produced from the ventricular zone, migrate to their destination, and incorporate into the existing neural network (e.g., López-García, Molowny, García-Verdugo, & Ferrer, 1988; Pérez-Cañellas & García-Verdago, 1996; Font, Desfílís, Pérez-Cañellas, & García-Verdugo, 2001).

The doublecortin marker has a distinct advantage over traditional exogenous markers of neurogenesis in that it only labels new neurons (Balthazart et al., 2008; Brown et al., 2003; Rao & Shetty, 2004). Previous studies in mammals and birds have found that doublecortin-labeled cells mostly exhibited a migratory morphology (Yang et al., 2004), expressed other markers indicative of immature neurons (Rao & Shetty, 2004; Yang et al., 2004), and did not coexpress with markers for astrocytes (Brown et al., 2003; Rao & Shetty, 2004; Yang et al., 2004), microglia (Yang et al., 2004), or oligodendrocytes (Brown et al., 2003; Rao & Shetty, 2004). Further, recent studies in lizards have confirmed that, within the cortical regions, doublecortin colabels precisely with other markers indicative of newly divided cells (Delgado-Gonzalez et al., 2011). Also, because doublecortin is endogenously expressed, it is not affected by variables associated with exogenous markers such as the amount or timing of injection (Greenough, Cohen, & Juraska, 1999; Gould & Gross, 2002), and avoids potential problems of toxicity at high doses (Cameron & McKay, 2001; Kolb, Pedersen, Ballermann, Gibb, & Whishaw, 1999).

In lizards, doublecortin expression in new neurons is known to occur anywhere from 7 to 90 days after a neuron is born, depending on the species (Delgado-Gonzalez et al., 2011; López-García, Molowny, García-Verdugo, Martinez-Guijarro, & Bernabeu, 1990; Marchioro et al., 2005; Ramírez-Castillejo, Nacher, Molowny, Ponsoda, & López-García, 2002). Because we housed individuals in their respective treatment groups for 5 months, well beyond 90 days, we were assured that we marked immature neurons that were produced exclusively during the timeframe of the treatment. It is also important to note that, in mammalian systems, once new neurons start expressing neuronal nuclei (NeuN), which is usually associated with fully mature neurons (Mullen, Buck, & Smith, 1992), expression of doublecortin ceases, even though there is a small window when both doublecortin and NeuN are coexpressed (Brown et al., 2003). Therefore, our analysis of neurogenesis likely only concerns the early immature stage of neuronal life.

Although doublecortin has been found primarily in areas of the adult mammalian brain known to exhibit neurogenesis, its expression has also been found in areas in which new neurons have not traditionally been observed (e.g., Kim, Peregrine, & Arnold, 2006). This has been suggested to be due to (a) low levels of neurogenesis occurring in areas of the brain not previously thought to undergo neurogenesis (e.g., Bernier, Bedard, Vinet, Levesque, & Parent, 2002; Magavi, Leavitt, & Macklis, 2000), or (b) migration of new neurons to or through areas of the brain not known to exhibit neurogenesis (e.g., Arvidsson, Collin, Kirik, Kokaia, & Lindvall, 2002). Nacher, Crespo, and McEwen (2001) also suggested that differentiated, adult neurons may have the capacity to

Figure 1. (a) Nissl visualization for total number of neurons; (b) new neurons were visualized with a marker for the endogenous expression of doublecortin. Both images were taken at 1000× magnification and scale bars represent 20 μm.
express doublecortin during changes in axon or dendrite growth or synaptogenesis, but they had no firm data supporting this conclusion. Klempin, Kronenberg, Cheung, Kettenmann, and Kempermann (2011) found that doublecortin-expressing cells in the possibly nonneurogenic periformal cortex of mice were postmitotic; however, within the hippocampus, they also found that cells that were positive for doublecortin strictly exhibited an immature phenotype and immature physiology. Brown et al. (2003) found that, within the rat dentate gyrus, cells that colabeled for doublecortin, NeuN, and bromodeoxyuridine (BrDU) were primarily found during the time period in which cells were transitioning from an immature phenotype to an adult phenotype. This suggests that coexpression of doublecortin and NeuN may be a product of ontogeny, during the time period when immature neurons are transitioning into adult neurons. Balthazart et al. (2008) also found that, in canaries, changes in immature and more mature cell morphology tracked the percentage of cells that colabeled for doublecortin and BrdU. Most important, using BrdU and doublecortin led to the same qualitative results when the effects of various treatments on adult neurogenesis were assessed (Couillard-Despres et al., 2005). Therefore, evidence suggests that doublecortin is a reliable marker for immature, migrating neurons, at least within the hippocampus and hippocampal homologues, in mammals, birds, and reptiles.

To visualize doublecortin, sections were washed in tris (hydroxymethyl)aminomethane-buffered saline (TBS), incubated in 30% hydrogen peroxide plus TBS (1:50) at room temperature for 30 min, washed in TBS, incubated in blocking buffer (normal horse serum, 1:33.3; Triton X-100, 1:39.8; and TBS) at room temperature for 30 min, and then incubated in ant doublecortin antibody plus blocking buffer (1:200; Santa Cruz Biotechnology, Santa Cruz, CA; SC-8066) overnight (approx. 18 hr) at 4 °C. The following day, sections were washed in TBS, incubated in biotinylated horse antigoat antibody in blocking buffer (1:200; Vector Laboratories, Burlingame, CA; BA-9500) at room temperature for 2 hr, washed in TBS, incubated in a VECA STAIN Elite ABC kit (Vector Laboratories, PK-6100) at room temperature for 1 hr, washed in TBS, reacted with diaminobenzidine + nickel kit (DAB-Ni; Vector Laboratories, SK-4100) at room temperature for 1 min and again washed in TBS and mounted on slides. We also performed a negative control to account for nonspecific binding of the secondary antibody. To do so, we repeated this protocol, but replaced the ant doublecortin antibody with TBS during overnight incubation. The elimination of the antibody suppressed staining, thus indicating that our protocol was specifically staining cells expressing doublecortin.

The Reptilian Brain

In the reptilian brain, the medial and dorsal cortices are thought to be homologous to the isocortex and the hippocampus of other vertebrates (Butler, 1994; Reiner, 1991, 1993; Striedter, 1997). Although the cortical regions do not exhibit columnar organization like the mammalian isocortex, and are composed of three layers rather than the mammalian six-layered isocortex, there are numerous homologies in the structures. For instance, neuronal types, neuronal morphologies, electrophysiological characteristics, types of neurotransmitters, and thalamic input all appear to have been, for the most part, conserved through the vertebrate lineages (e.g., Reiner, 1991, 1993). The medial cortex, in particular, has been shown to exhibit neuronal types and laminar structure similar to the dentate gyrus of the hippocampus (Luis de la Iglesia & López-García, 1997; Luis de la Iglesia, Martínez-Guijarro, & López-García, 1994), with axonal projections similar to hippocampal mossy cells (López-García, & Martínez-Guijarro, 1988). Also, the ventricular zone, the area of the brain adjacent to the brain ventricles and the location of the population of adult proliferating cells, has been conserved across amniotic vertebrates, as have the cell types that make up the ventricular zone (e.g., García-Verdugo et al., 2002). Although neurogenesis in mammals appears to be more spatially restricted than nonmammals, in all amniotic vertebrates, new neurons are generated in the ventricular zone, then migrate and incorporate into the existing neural network.

In terms of functional homologies, the medial and dorsal cortices of reptiles have been implicated in spatial processing, as lesions to either region impair spatial abilities (Grisham & Powers, 1990; López, Vargas, Gomez, & Salas, 2003; Pettrillo, Ritter, & Schade Powers, 1994; Reiman Avigan & Schade Powers, 1995; Rodríguez et al., 2002), thus sharing similar functions to the hippocampus in other vertebrates. However, because the evolution of the structure of the isocortex and hippocampus is more complex in mammals, functionality and processing abilities in mammals are likely more complex as well (e.g., Reiner, 1991, 1993).

Brain Analysis

We used standard stereological methods (StereoInvestigator, Microbrightfield, Inc., Williston, VT; microscope Leica M4000B) optimized for this species to measure volume, total number of neurons, and neurogenesis rates of the medial and dorsal cortices (LaDage, Riggs et al., 2009). First, on each section, the cortical regions and remainder of the telencephalon were measured in their entirety and volumes were estimated with the Cavalieri procedure (Gundersen & Jensen, 1987). Cortical areas were measured with a 200-μm grid and the remainder of the telencephalon was measured with a 300-μm grid. The left and right hemispheres were both measured and summed to produce the given values, as there were no significant differences between left and right cortical or telencephalon volumes.

Next, total neuron counts were performed on each Nissl-stained section with an optical fractionator procedure with a counting frame of 30 × 30 μm, a 250-μm grid, and a 5-μm dissector height. Because our sectioning was relatively thick (40 μm), this precluded counting cells within the cell layer of the cortices. Following neuronal identification guidelines, we identified neurons as cells with one or two darkly stained nucleoli, clear nucleoplasm, and lightly colored dendritic processes extending from the cell body (e.g., Barnea & Nottebohm, 1994; Pérez-Cañellas & García-Verdugo, 1996; Sherwood et al., 2006). New neuron counts were performed on every doublecortin-stained section similarly to total neuron counts, except because there were relatively fewer new neurons than total neurons, new neurons (i.e., all cells expressing doublecortin) were counted exhaustively with a counting frame of 70 × 70 μm, a 70-μm grid, and a 5-μm dissector height. All doublecortin-positive cells were counted, including the fusiform migrating neurons perpendicular to the ventricular zone, and the more mature, spherical phenotype with more extensive dendritic branching (e.g., Pérez-Cañellas & García-Verdugo, 1996). Again,
the left and right hemispheres were both measured and summed to produce the given values, as there were no significant differences between left and right total and new neuron counts.

Statistical Methods

Distance moved over 8 hr, volume of each cortical region, total number of neurons within each region were analyzed with a repeated-measures ANOVA to account for sibling relationships. For cortical-volume analyses, volume of the remainder of the telencephalon was used as a covariate to account for overall changes in brain volume. For the total number of neurons, volume of the cortical region of interest was used as a covariate to account for overall volumetric changes (essentially a measure of density). For the number of immature neurons, the total number of neurons in that cortical region was used as a covariate to account for overall neuron number (essentially a measure of the proportion of new to total neurons). We also report all analyses without covariates. We considered all results to be statistically significant if \( p < .05 \).

Results

For the movement data, we found a significant effect of enclosure size on distance moved over 8 hr; males in larger enclosures traveled greater distances than did their siblings in smaller enclosures (\( F_{1,5} = 6.693, p = .041 \); Figure 2). There was no significant effect of territorial status on distance moved (\( F_{1,5} = 1.082, p = .338 \)) and an interaction between enclosure size and territorial status was also nonsignificant (\( F_{1,5} = 1.087, p = .337 \)).

The volumes of the medial and dorsal cortices were not significantly different between territorial and nonterritorial males (medial, \( F_{1,5} = 5.593, p = .064 \); dorsal, \( F_{1,5} = 4.036, p = .101 \)) or between males in large versus small enclosures (medial, \( F_{1,5} = 0.158, p = .707 \); dorsal, \( F_{1,5} = 0.301, p = .607 \)), and the interaction between territorial status and enclosure type was not significant (medial, \( F_{1,5} = 0.708, p = .439 \); dorsal, \( F_{1,5} = 0.390, p = .560 \)). Similar results were found when the covariate was removed from the analysis (territoriality medial, \( F_{1,6} = 2.087, p = .199 \); territoriality dorsal, \( F_{1,6} = 2.280, p = .182 \); enclosure size medial, \( F_{1,6} = 0.280, p = .616 \); enclosure size dorsal, \( F_{1,6} = 1.833, p = .225 \); interaction medial, \( F_{1,6} = 0.730, p = .426 \); interaction dorsal, \( F_{1,6} = 0.319, p = .593 \)).

The total number of neurons in the medial and dorsal cortices was not significantly different between territorial and nonterritorial males (medial, \( F_{1,5} = 0.250, p = .638 \); dorsal, \( F_{1,5} = 1.657, p = .254 \)), between males in large versus small enclosures (medial, \( F_{1,5} = 0.361, p = .574 \); dorsal, \( F_{1,5} = 0.132, p = .731 \)), or between territorial status and enclosure type (medial, \( F_{1,5} = 0.583, p = .480 \); dorsal, \( F_{1,5} = 0.012, p = .917 \)). Similar results were found when the covariate was removed from the analysis (territoriality medial, \( F_{1,6} = 2.013, p = .206 \); territoriality dorsal, \( F_{1,6} = 4.482, p = .079 \); enclosure size medial, \( F_{1,6} = 0.208, p = .664 \); enclosure size dorsal, \( F_{1,6} = 0.158, p = .705 \); interaction medial, \( F_{1,6} = 0.363, p = .569 \); interaction dorsal, \( F_{1,6} = 0.175, p = .691 \)).

The number of new immature neurons in the medial and dorsal cortices was not significantly different between territorial and nonterritorial males (medial, average proportion of new neurons = 0.053 nonterritorial, 0.137 territorial, \( F_{1,5} = 0.373, p = .111 \); dorsal, average proportion of new neurons = 0.032 nonterritorial, 0.074 territorial, \( F_{1,5} = 4.032, p = .101 \)) or between males in large versus small enclosures (medial, average proportion of new neurons = 0.111 large enclosure, 0.079 small enclosure, \( F_{1,5} = 0.532, p = .498 \); dorsal, average proportion of new neurons = 0.062 large enclosure, 0.044 small enclosure, \( F_{1,5} = 0.334, p = .588 \)). We did not detect a significant interaction effect in the dorsal cortex (\( F_{1,5} = 1.689, p = .250 \); see Figure 3a) but we did detect a significant interaction effect in the medial cortex (\( F_{1,5} = 12.044, p = .018 \); see Figure 3b). Fisher’s least significant difference tests indicated that territorial males in larger enclosures had significantly more new immature neurons than their siblings in smaller enclosures (average proportion of new neurons = 0.178 large enclosures, 0.095 small enclosures, \( p = .010 \)). Territorial males in larger enclosures also had significantly more new neurons than nonterritorial males in larger enclosures (average proportion of new neurons = 0.043 nonterritorial males, 0.178 territorial males, \( p = .027 \); see Figure 3b). Similar results were found when the covariate was removed from the analysis (territoriality medial, \( F_{1,6} = 2.950, p = .137 \); territoriality dorsal, \( F_{1,6} = 3.019, p = .199 \); enclosure-size dorsal, \( F_{1,6} = 1.211, p = .313 \); enclosure-size medial, \( F_{1,6} = 6.457, p = .044 \); interaction medial, \( F_{1,6} = 25.253, p = .002 \); interaction dorsal, \( F_{1,6} = 0.446, p = .080 \)). Even though the overall effect of the enclosure size on medial cortex was significant, a significant interaction followed by the Tukey post hoc test indicated that only the territorial males in the larger enclosures had significantly more new neurons in their medial cortices than the territorial males in the smaller enclosures (\( p < .05 \)), but such differences were nonsignificant for nonterritorial males.

Discussion

We found that neurogenesis rates in the medial cortex of side-blotched lizards differ depending on an interaction between territorial status and spatial-area use. Territorial males in larger enclo-
sures had increased medial cortex neurogenesis rates compared with their siblings housed in smaller enclosures, as well as higher medial cortex neurogenesis than nonterritorial males in larger enclosures.

These results did not fully conform to our predictions. Because an increase in spatial-area use has been shown to correlate with an increase in brain attributes, we predicted that males, regardless of territorial status, would demonstrate an increase in cortical attributes due to increased physical activity and increased spatial learning and memory; this was not the case for nonterritorial males when we accounted for change in the total number of neurons. Nonterritorial males had similar rates of neurogenesis when compared with their siblings in smaller enclosures, despite significantly increased overall physical activity levels and potentially increased demands on spatial learning and memory. One possible explanation is that it may be less important for nonterritorial males to encode additional spatial information when moving from a smaller to larger enclosure. If there is no benefit, in terms of survival or reproductive success, to remembering a particular spatial area, then alterations in spatial-area use would not be significant differences in number of doublecortin-positive neurons between territorial males in large enclosures and territorial males in small enclosures, as well as significant differences between territorial males in large enclosures and nonterritorial males in large enclosures.

![Figure 3](image)

**Figure 3.** Number of doublecortin-positive neurons between territorial (orange and blue morphotypes) and nonterritorial (yellow morphotypes) males in small and large enclosures (open circles, nonterritorial; closed circles, territorial) in (a) the dorsal cortex and (b) the medial cortex. The asterisk (*) indicates statistically significant differences in number of doublecortin-positive neurons between territorial males in large enclosures and territorial males in small enclosures, as well as significant differences between territorial males in large enclosures and nonterritorial males in large enclosures.
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sures exhibit increased neurogenesis (Kempermann et al., 1997). Other factors, such as chronic stress and increased corticosterone levels, both of which have been shown to depress levels of neurogenesis (Gould & Tanapat, 1999; Lupien, McEwen, Gunnar, & Heim, 2009; Montaron et al., 2006). Although we did not measure corticosterone levels in these males, it may be possible that nonterritorial males, when moved from smaller to larger enclosures, perceive larger enclosures as more stressful, and this may be reflected in increased corticosterone levels. Variation in physiological responses has been shown between individuals with differing mating behaviors and strategies. For instance, previous studies have found that nonterritorial male tree lizards (Urosaurus ornatus) appear to be more physiologically sensitive to stressful contexts in that, in the face of stressful situations, they increase circulating levels of corticosterone compared with their territorial counterparts (Knapp & Moore, 1996; Moore, Hews, & Knapp, 1998). Thus, it may be that our nonterritorial males were also more physiologically sensitive to stress, and the increased stress and corticosterone levels in larger enclosures overrode the positive effects of increased spatial-area use on neurogenesis. Future studies could potentially elucidate the relative contributions of particular factors (e.g., physical movement, hormonal responses) on rates of neurogenesis.

Territorial males in larger enclosures, however, did show an increase in neurogenesis rates when compared with their siblings in smaller enclosures, supporting our first prediction and the results of previous studies. Territorial males had increased levels of physical activity and likely increased demands on spatial processing when compared with their siblings in smaller enclosures, and these factors have been shown to increase rates of neurogenesis. This particular result more closely conforms to expectations based on previous studies. For example, rodents moved from standard laboratory enclosures to larger and more spatially complex enclosures exhibit increased neurogenesis (Kempermann et al., 1997).

In this study, we only considered the effects due to increased spatial-area use, rather than spatial complexity; it may be that an increase in spatial complexity can produce additional variation in the cortical regions in the side-blotched lizard.

When looking at the interaction between territorial status and spatial-area use, we found that territorial males in larger enclosures had increased rates of neurogenesis when compared with nonterritorial males in larger enclosures, which supported our predictions. These results suggest that territorial and nonterritorial males may have differing responses to variability in spatial-area use as spatial area is increased; territorial males appear to be more sensitive to variation in the spatial environment.

What is less clear is the mechanism driving this difference. Previous studies on adult rodents have demonstrated that voluntary running increases neurogenesis and other neuronal attributes (e.g., Farmer et al., 2004; Van Praag, Christie et al., 1999, Van Praag, Kempermann et al., 1999), thus we expected that physical activity and neurogenesis would increase in both morphotypes in larger enclosures. We did find that males, regardless of territorial status, increased physical activity in larger enclosures when compared with their brothers in smaller enclosures. Also, we did not detect significant differences in physical activity between territorial and nonterritorial males in larger enclosures. Thus, the mechanism that drives the difference in neurogenesis between the morphotypes in larger enclosures remains elusive. It may be that territorial males are more sensitive to alterations in physical activity levels between smaller and larger enclosures, whereas nonterritorial males do not respond to increased activity levels. Or, as mentioned above, nonterritorial males may be subject to counteracting effects, such as stress, that may mask increased neurogenesis levels due to physical activity (e.g., Stranahan, Khalil, & Gould, 2006).

Alternatively, males that hold territories may have increased demands on spatial-processing ability because of the need to remember spatial-area use for territorial defense. Previous studies have found that individuals or species that have increased demands on their spatial-memory ability, in an ecological context, tend to have larger hippocampal attributes (Sherry, 2006). For example, some food-caching species rely heavily on spatial memory to recover cached food items. These species also have larger hippocampi than closely related species that do not cache and retrieve food (Healy & Krebs, 1996; Krebs, Sherry, Healy, Perry, & Vaccarino, 1989; Lucas, Brodin, de Kort, & Clayton, 2004; Sherry, Vaccarino, Buckenham, & Herz, 1989). Similarly, male meadow voles (Microtus pennsylvanicus), which maintain large home ranges relative to female home ranges, have larger hippocampi than females, likely due to the increased demands on their spatial-memory load (Jacobs, Gaulin, Sherry, & Hoffman, 1990).

Further, territorial morphs of male side-blotched lizards have larger dorsal cortices (reptilian hippocampal homologue) than do nonterritorial males, possibly due in part to increased demands on spatial processing in the context of territorial defense (LaDage, Riggs et al., 2009). Thus, territorial males may rely more heavily on spatial learning and memory in the context of territorial defense. Because these males garner reproductive success via competitive exclusion of other males on their territories, it would be beneficial to these males to encode and recall the spatial boundaries of their territories. As nonterritorial males do not rely upon the defense of a particular spatial area, there may be no selective advantage to remembering a spatial area in the face of increased spatial-area use. Therefore, the two morphotypes may have evolved differential sensitivity to alterations in the spatial environment. Finally, another potential mechanism may be maternally modulated effects, such as differential yolk estrogen deposition, which have been shown to vary as a function of progeny genotype (Lancaster, McAdam, Wingfield, & Sinervo, 2007). It may be that differential deposition of estrogen could have varying effects on the neuronal phenotype as well, but this has yet to be determined. Overall, the underlying mechanisms surrounding the differences we have found still need to be elucidated.

It is interesting to note that we found the interaction effect in neurogenesis rates only in the medial cortex and not in the dorsal cortex. The medial cortex has been shown to be involved in spatial learning, and lesions to this area in turtles induce deficits in spatial learning (López et al., 2003; Rodríguez et al., 2002). Thus, an
increase in neurogenesis rates in the medial cortex, when faced with an increase in spatial information processing, may not be surprising. However, we did not find such a difference in the dorsal cortex, an area of the brain involved in processing spatial maps in reptiles (Grisham & Powers, 1990; Petrillo et al., 1994; Reiman Avigan & Schade Powers, 1995). Unfortunately, there is a paucity of studies that actually examine neurogenesis in reptiles and, of those studies, very few actually examine the functional significance of reptilian neurogenesis. Of the studies examining new neuron distribution in various brain regions in reptiles, some have found differences in the amount of neurogenesis between the two brain regions: More new neurons appear to be located in the medial than in the dorsal cortex (Font et al., 2001). In addition, the functional relevance of neurogenesis may differ in these brain areas, potentially contributing to the differences we detected. The cortical disparity in the production of new neurons and the function of these new neurons may partially explain why we did find differences in the medial, but not the dorsal, cortex.

It is important to mention that we could not separate cell-proliferation rates from neuron-survival rates in our study, as doublecortin labeling generates a combined measure of production and survival (Rao & Shetty, 2004). Previous studies have found that both cell-proliferation rates and new neuron survival can be affected by an increase in spatial area (Kempermann et al., 1997; Kempermann, Brandon, & Gage, 1998; Kronenberg et al., 2003), but the effects of environmental enrichment, including learning, on new neuron proliferation and survival may be confined to a critical period (Döbrössy et al., 2003; Tashiro, Makino, & Gage, 2007). In fact, spatial learning was shown to induce complex physiological processes, including alterations in cell proliferation, survival, and apoptosis (Dupret et al., 2007). Therefore, in our study, it is possible that some subtle differences, specifically in neuron proliferation or survival rates, may have gone undetected. Regardless, doublecortin labeling has been shown to track the percentage of cells that also label for BrdU, the traditional marker used to quantify cell proliferation and survival rates (Balthazart et al., 2008; Brown et al., 2003; Couillard-Despres et al., 2005; Rao & Shetty, 2004). Thus, although we could not distinguish cell proliferation or survival rates, doublecortin provides a reliable measure of overall neurogenesis rates and we did find differences between territorial and nonterritorial males in larger enclosures.

Although we found differences in neurogenesis rates, we did not find statistically significant differences among males in cortical volumes and total neuron numbers. This was somewhat surprising, as differences between morphotypes in the volume of the dorsal cortex have been shown in wild-caught animals in this species (LaDage, Riggs et al., 2009)—territorial males had larger dorsal cortices than nonterritorial males. However, there may be several explanations for these results. First, our previous study involved wild-caught animals that were sacrificed immediately after being removed from the wild (LaDage, Riggs et al., 2009) whereas, in this study, animals were born and raised in captivity. Captivity represents a severely restricted spatial environment, thus leading to decreased spatial-area use and decreased need to integrate the location of territorial neighbors, when compared with the wild. As such, we would expect that animals living in captivity might experience restricted physical movement and spatial-processing experiences when compared with conspecifics in the wild, which may then be reflected in hippocampal attributes (Barnea & Nottebohm, 1994; Day, Guerra, Schlinger, & Rothstein, 2008; LaDage, Roth et al., 2009; Smulders, Casto, Nolan, Kettersen, & DeVoogd, 2000; Van Praag et al., 2000). Likewise, in previous studies, captive dark-eyed juncos (Junco hyemalis), brown-headed cowbirds (Molothrus ater obscurus), and mountain chickadees (Poecile gambeli) have been shown to have smaller hippocampal volumes than wild-caught counterparts (Day et al., 2008; LaDage, Roth et al., 2009; Smulders et al., 2000). Therefore, in our study, animals raised in captivity may have had decreased cortical volumes when compared with their wild conspecifics because of a restricted spatial environment that decreases physical movement and spatial-processing opportunities.

Alternatively, it may be that lizards in our study did not have sufficient spatial-area use, even in the larger enclosures, to induce changes in cortical volumes. Similar to the captivity effect, the difference in spatial area between smaller and larger enclosures may not have been disparate enough to cause increased physical stimulation or spatial-learning and memory experiences, thus not inducing changes in the cortical volumes. Previous studies found that, when raising birds in captivity, animals must have a sufficient number of spatial-processing experiences before those experiences are reflected in the volume of the hippocampus (Clayton, 2001). However, in previous studies, an increase in the spatial area of an enclosure can cause changes in brain attributes, even within the artificial confines of the laboratory. For instance, mice in standard laboratory enclosures (48 × 26 cm) had decreased levels of neurogenesis compared with mice in larger enclosures (86 × 76 cm; for a review, see Van Praag, Kempermann, & Gage, 2000). This represents an increase in spatial-area use of 5× the standard cage enclosures, but even this increase in spatial area is far below their natural home range of ~300 m² (Mikesic & Drickamer, 1992). Our increase in spatial area was 25× the standard cage enclosure, thus we assumed that an increase of this magnitude would be sufficient to induce changes in cortical attributes. Finally, it may be that cortical volumes and total neuron numbers do not exhibit the same trajectory as the number of new neurons, thus we did not have statistical power to elucidate differences in those variables, if differences existed. Our sample size may have been large enough to detect differences in number of new neurons, but not large enough for those differences, if they existed, to be detected in cortical volume and total number of neurons.

An effect of territorial status and spatial use on neurogenesis but not total cell number may seem counterintuitive, considering that increased neurogenesis in territorial males in larger enclosures should translate into more total neurons. However, our findings may conform to some extent to trends seen in previous studies. For example, some studies have found that total neuron numbers are far less sensitive to spatial-processing experiences, appear to be more stable across spatial contexts than hippocampal volume and neurogenesis, and, in some cases, may be controlled by mechanisms less responsive to environmental conditions (Barnea & Nottebohm, 1994; Clayton, 2001; Cristol et al., 2003; LaDage, Roth et al., 2009; Pravosudov & Clayton, 2002; Roth, LaDage, Freas, & Pravosudov, 2012; Valero et al., 2011). Moreover, it has been shown that other neurobiological processes in the brain are affected by differential spatial processing. For example, there appears to be an interrelationship among proliferation, survival, and death of new neurons. Thus, in the face of increased spatial-processing demands, neurogenesis rates may increase, but apopto-
sis of older neurons may also increase, thereby maintaining a constant net amount of total neuron numbers (Dupret et al., 2007). Thus, perhaps our findings suggest that the relationship between total neuron number and neurogenesis is more complex than it may at first appear.

Ultimately, it appears that, in the side-blotched lizard, there is a relationship between life-history characteristics, in this case territoriality status, and alterations in the spatial environment. These findings have given us the opportunity to refine our knowledge and redefine the importance of variation in the spatial environment on neurogenesis. We must consider the natural history and different selection pressures of particular species, morphotypes, and so forth before assuming that all individuals respond similarly to alterations in the spatial environment. Additional studies examining the hormonal underpinnings, as well as controlling for other factors important to the modulation of neurogenesis (e.g., stress, spatial complexity), would provide additional details to aid our understanding of neurogenesis in the context of varying spatial environments and life-history characteristics.

References


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