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Source: *Journal of Herpetology*, 51(2):258-262.

Published By: The Society for the Study of Amphibians and Reptiles

DOI: <http://dx.doi.org/10.1670/16-056>

URL: <http://www.bioone.org/doi/full/10.1670/16-056>

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## Neuroanatomical Changes Related to a Changing Environment in Lesser Earless Lizards

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**ABSTRACT.**—How do changes in the environment affect the brain? More specifically, how do different physical environments shape areas of the brain that facilitate the contextual perception of animals' surroundings? We evaluated the relationship between rapid habitat change and the volume of the medial, lateral, and dorsal cortices, in two populations of wild male and female Lesser Earless Lizards (*Holbrookia maculata*). These populations inhabit neighboring, but physically distinct, environments in southern New Mexico: White Sands and dark soil habitats. The White Sands habitat is a geologically recent environment that formed over the last 2,000–7,000 yr. There is a stark physical contrast between White Sands and the surrounding typical Chihuahuan desert habitat that is characterized by dark soil, denser vegetation, and higher competitor and predator species abundances. We observed differences in the volumes of the medial cortex, an important neuroanatomical structure for navigation, but not in those of the dorsal or lateral cortices, between lizards inhabiting the White Sands and dark soil habitats. Male and female lizards in the White Sands habitat had smaller medial cortices (relative to their overall brain size) compared to those in the dark soil environment. We also found sex differences in the volume of the medial cortex; in the dark soil habitat, males had larger medial cortices than females. In summary, we have uncovered differences in neuroanatomy between habitats and between sexes in a wild species of lizard, which may reflect neuroanatomical plasticity and/or rapid evolutionary change in response to a geologically recent environmental change.

**RESUMEN.**—¿Cómo afectan los cambios en el ambiente al cerebro? ¿Específicamente, cómo moldean los distintos ambientes físicos las áreas del cerebro que facilitan la percepción contextual del entorno de los animales? Evaluamos la relación entre cambios repentinos en el hábitat y el volumen de las cortezas mediales, laterales, y dorsales en dos poblaciones silvestres de machos y hembras de las lagartijas *Holbrookia maculata*. Estas poblaciones habitan ambientes vecinos, pero físicamente disímiles en el sur de Nuevo México, EUA: las Arenas Blancas y suelos oscuros. Las Arenas Blancas son un hábitat geológicamente reciente que se formó a través de los últimos 2,000–7,000 años. Hay un contraste físico severo entre las Arenas Blancas y el entorno típico del desierto de Chihuahua, el cual se caracteriza por suelos oscuros, vegetación densa, y densidades mayores de competidores y depredadores. Observamos diferencias en el volumen de la corteza medial, una estructura neuroanatómica importante para la navegación, pero no en la dorsal o lateral, entre lagartijas que moraban en los hábitats de suelo oscuro versus las que habitaban las Arenas Blancas. Las lagartijas hembras y machos de las Arenas Blancas tenían cortezas mediales más pequeñas (relativo al tamaño cerebral total) comparadas con las que habitaban en el ambiente de suelos oscuros. También encontramos diferencias en el volumen de la corteza medial entre los sexos; en el hábitat de suelos oscuros los machos tenían cortezas mediales más grandes que las hembras. Resumiendo, hemos descubierto diferencias en la neuroanatomía entre hábitats y sexos en un especie silvestre de lagartija, lo cual puede indicar plasticidad neuroanatómica y/o cambio evolutivo veloz en respuesta a un cambio ambiental geológicamente reciente.

Brain–environment interactions have been examined mostly in artificial laboratory settings with classic model species; however, studies on nonmodel species in the wild have provided a new understanding of how brain morphology is linked to the environment (e.g., Jacobs et al., 1990; Sherry et al., 1992; Day et al., 2001; Powell and Leal, 2012; Roth et al., 2012). In a rapidly changing world, the study of neuromorphological plasticity or microevolution in response to environmental shifts is essential (e.g., Roth and Pravosudov, 2009; Freas et al., 2013; Pravosudov et al., 2013). To increase understanding of the relationship between environmental change and brain morphology in a wild species, we examined the neuroanatomy of a lizard population that has experienced rapid adaptation to a novel habitat.

Lesser Earless Lizards (*Holbrookia maculata*) inhabit two contrasting habitats in New Mexico: the typical dark soil habitat of the Chihuahuan Desert and the novel White Sands habitat. The White Sands formation is geologically recent

(formed in the last 2,000–7,000 yr) and represents a dramatic environmental change. Compared to the surrounding dark soil habitat, the White Sands habitat has lighter-colored substrate, sparser vegetation, and lower overall species richness (Des Roches et al., 2011; Rosenblum and Harmon, 2011; Fig. 1). As a result, the White Sands population of *H. maculata* has diverged rapidly from the ancestral dark soil population in a number of traits, including coloration, morphology, mate preference, and diet (e.g., Rosenblum, 2006, 2008; Des Roches et al., 2011; Rosenblum and Harmon, 2011). Because of these environmentally driven changes in morphology and behavior, the White Sands system presents a rare opportunity to document how brain morphology may respond to changing environments.

Reptile cerebral cortices are divided into three major regions: the medial, dorsal, and lateral cortices. The medial cortex (Fig. 2), or hippocampus (the mammalian homolog), is a particular region of interest, because it is the area that undergoes the greatest amount of postnatal neurogenesis in the lizard telencephalon (Lopez-Garcia et al., 1984), and it responds to changes in the environment. Squamate reptiles, like many other taxa, possess spatial memory abilities (LaDage et al., 2012). The reptilian medial cortex, much like the mammalian hippocam-

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FIG. 1. Contrasting habitats occupied by different *H. maculata* ecomorphs: (A) dark soil and (B) White Sands.

pus, is an important neuroanatomical structure for navigation (Hoogland and Vermeulen-Van der Zee, 1987; Day et al. 2001). The positive relationship, in which there is a greater cell expanse in the medial cortex of species that inhabit more spatially complex and demanding habitats, has been reported in other lizards (Powell and Leal, 2012), birds (Roth et al., 2012), bats (Safi and Dechmann, 2005), fish (Shumway, 2008), and humans (Maguire et al., 2000). Because of the dramatic differences in the habitats inhabited by Lesser Earless Lizards, we hypothesized that there would be differences in the size of the medial cortex in the White Sands and dark soil ecomorphs. The White Sands habitat has less vegetation, reduced species richness, and abundance of competitors and predators (Des Roches et al., 2011; Rosenblum and Harmon, 2011). For example, White Sands contains about half of the amount of yucca trees and shrubs as compared to the surrounding Chihuahuan desert, and three species of lizards as compared to 35 species of reptiles in the

Chihuahuan desert. Therefore, we predicted that the White Sands lizards, living in a habitat with potentially lower spatial and biological complexity, would have smaller medial cortices, compared to their ancestral dark soil counterparts. We also examined adjacent brain areas, the dorsal and lateral cortices (Fig. 2) for lizards in contrasting habitats, as these areas may also be related to the ecological use of space (Day et al., 1999).

#### MATERIALS AND METHODS

**Sample Collection.**—We collected 18 adult *H. maculata* specimens, 9 from the White Sands habitat (4M : 5F; White Sands National Monument, Otero County, New Mexico) and 9 from the dark soil habitat (4M : 5F; Jornada Experimental Range, Don Ana County, New Mexico and White Sands Missile Range, Lincoln County, New Mexico), during the summer of 2013. We collected lizards with the use of a common noosing technique. To obtain brain samples, we rapidly decapitated the lizards and made incisions in the skull to expose the brain. We then incubated the brains in a 4% paraformaldehyde solution for approximately 24 h. Following this fixation process, we dissected out the brains from the lizards' skulls, and then cryoprotected them in a 30% sucrose–azide solution at 4°C, until we had returned them to the laboratory at the University of California, Berkeley. In the laboratory, the samples were frozen at –80°C until further processing the following month. To confirm that animals were adults, we examined the size of gonads (large indicated reproductively mature, versus tiny/regressed) and snout-to-vent length (SVL) (>44 mm is indicative of an adult; Rosenblum, 2008).

**Sectioning and Staining.**—We sectioned whole brains coronally into 30- $\mu$ m-thick sections, on a CM3050S cryostat (Leica Microsystems, Wetzlar, Germany) at –20°C. Then we mounted every fifth section directly on silane-coated slides and stained them using a standard cresyl violet staining protocol (Day et al., 2001; Powell and Leal, 2012). Briefly, we incubated the slides, with the

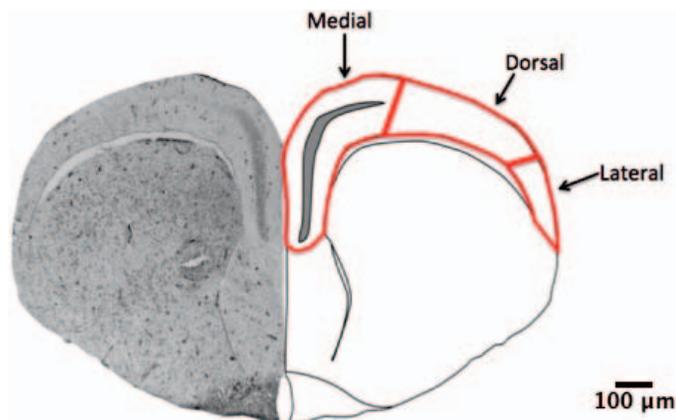


FIG. 2. Representative image of a coronal section (left) and a stylized section depicting the medial, dorsal, and lateral cortices (right; outlined in red).

TABLE 1. The main effect of sex and habitat on the relative volumes of the medial, dorsal, and lateral cortices (results from the two-way ANOVAs).

Brain area	Experimental factor	Degrees of freedom (df)	Significance (P)	F-test	Partial eta-squared
Medial	Sex	1	0.027*	6.221	0.324
	Habitat	1	0.031*	5.847	0.310
	Sex * Habitat	3	0.77	3.690	0.224
Dorsal	Sex	1	0.729	0.126	0.010
	Habitat	1	0.226	1.616	0.111
	Sex * Habitat	3	0.746	0.110	0.008
Lateral	Sex	1	0.184	1.964	0.131
	Habitat	1	0.529	0.418	0.031
	Sex * Habitat	3	0.715	2.053	0.136

sections mounted, in 100% chloroform for 10 min and then progressively rehydrated them in 100%, 95%, 90%, and 70% ethanol for 3 min each. Then, we incubated the slides in deionized water for 5 min, after which we stained them with 0.1% cresyl violet for 10 min. The mounted sections were again dehydrated sequentially in 70%, 90%, 95%, and then 100% ethanol. Finally, we placed the mounted sections in xylene for 5 min and cover-slipped them with Krystalon mounting medium (Thermo-Fisher Scientific, Inc., Waltham, Massachusetts).

*Image Capture and Analysis.*—With the use of a double-blind technique, we visualized the brain sections under an Axioimager M1bright field microscope (Carl Zeiss Mfg., Oberkochen, Germany) and then photographed them with an Axiocam MRm at  $\times 25$  magnification (Carl Zeiss Mfg.). Then, with the use of Photoshop CS6 (Adobe Systems, Inc., San Jose, California), we traced the area of the left side of the medial, dorsal, and lateral cortices for each brain, starting from first detection of the areas until final detection, as well as the area of the entire left hemisphere of the brain (Fig. 2). We measured these outlined areas of interest with the use of ImageJ (National Institutes of Health; 64-bit mode). To control for overall brain size, we divided the medial, dorsal, and lateral measurements by the size of the entire left hemisphere of the brain. Brain volumes were estimated with the Cavalieri procedure (Gundersen and Jensen, 1987). The medial, dorsal, and lateral cortices, and the entire left hemisphere of the brain also were measured with a 200- $\mu$ m grid.

*Statistical Analyses.*—First, we used a Student's *t*-test to examine whether populations differed in SVL. Then, we used three two-way ANOVAs to examine the influence of sex (male, female), habitat (dark soil, White Sands), and their interactions on the 1) medial, 2) dorsal, and 3) lateral cortex volumes. If the models were significant ( $P < 0.05$ ), we conducted post hoc Tukey's honestly significant difference (HSD) multiple-comparisons tests to reveal the means that were significantly different from each other. To examine the magnitude of differences between groups, we also measured effect size with the use of a partial eta-squared test. A small effect size was considered when  $n_p^2$  was 0.01–0.08, and moderate and large effect sizes were considered when  $n_p^2$  was 0.09–0.24 and  $\geq 0.25$ , respectively (Sullivan and Feinn, 2012; Lakens, 2013).

## RESULTS

We confirmed all animals to be adults. All animals had large gonads, indicative of sexual maturity. There were no significant differences between population SVLs ( $P = 0.168$ ); mean  $\pm$  SD SVL = 49.83  $\pm$  5.64 mm (dark soil) and 52.83  $\pm$  4.58 mm (White Sands).

TABLE 2. Analyses of the interactions between sex and habitat on the relative volumes of the medial, dorsal, and lateral cortices (results from Tukey's HSD post hoc tests).

Sex * Habitat	Sex * Habitat	Medial (P)	Dorsal (P)	Lateral (P)
White Sands female (WS F)	ds F	0.983	0.902	0.939
	WS M	0.975	1.000	1.000
	ds M	0.016*	0.649	0.472
Dark soil female (ds F)	WS F	0.983	0.902	0.939
	WS M	1.000	0.920	0.953
	ds M	0.041*	0.964	0.254
White Sands male (WS M)	WS F	0.975	1.000	1.000
	ds F	1.000	0.920	0.953
	ds M	0.045*	0.693	0.502
Dark soil male (ds M)	WS F	0.016*	0.649	0.472
	ds F	0.041*	0.964	0.254
	WS M	0.045*	0.693	0.502

Our models revealed that the relative volume of the medial cortex depended on sex ( $F_{1,17} = 6.221$ ,  $P = 0.027$ ) and habitat ( $F_{1,17} = 5.847$ ,  $P = 0.031$ ; Table 1). The effect size was large for both sex ( $n_p^2 = 0.324$ ) and habitat ( $n_p^2 = 0.310$ ). Specifically, dark soil males had larger medial cortices than dark soil females ( $P = 0.041$ ), White Sands males ( $P = 0.045$ ), and White Sands females ( $P = 0.016$ ; Table 2; Fig. 3). The relative volume of the dorsal and lateral cortices did not depend on sex, habitat, or their interaction (Table 1; Fig. 3). Although there were no significant differences found for the lateral cortex there was a medium effect size for sex ( $n_p^2 = 0.136$ ) and the interaction of sex and habitat ( $n_p^2 = 0.131$ ) in this brain region.

## DISCUSSION

We set out to determine whether differences in neuroanatomical structures were associated with habitat differences for diverging populations of wild lizards. Specifically, we examined differences in the volume of the medial, dorsal, and lateral cortices in *H. maculata* populations inhabiting two dramatically different environments: the typical dark soil habitat of the Chihuahuan Desert and the geologically recent White Sands formation. We found predictable differences in the neuroanatomy of the medial cortex between the ecomorphs and between the sexes. This finding is compelling because it suggests that structural aspects of the brain can change in response to habitat changes in wild populations of animals.

We found smaller medial cortex volumes in White Sands lizards, consistent with our initial predictions based on habitat differences. Lizards inhabiting what has been characterized as a less complex environment in terms of less vegetation, reduced species richness, and abundance of competitors and predators (Des Roches et al., 2011; Rosenblum and Harmon, 2011) had a smaller medial cortex, and this difference appears to be driven by a smaller medial cortex in the White Sands males (Fig. 3). Differences in the females were not as pronounced between the habitats and not statistically significant. Male *H. maculata* generally have larger home range sizes and traverse greater distances of the landscape than females (Jones and Droge, 1980). Roth et al. (2006) report that in the snake species, *Agistrodon piscivorus*, male medial cortex volume is positively correlated with patterns of spatial use. Therefore, perhaps a shift in the environment of male *H. maculata* may disproportionately affect the male neuroanatomy involved in spatial processing.

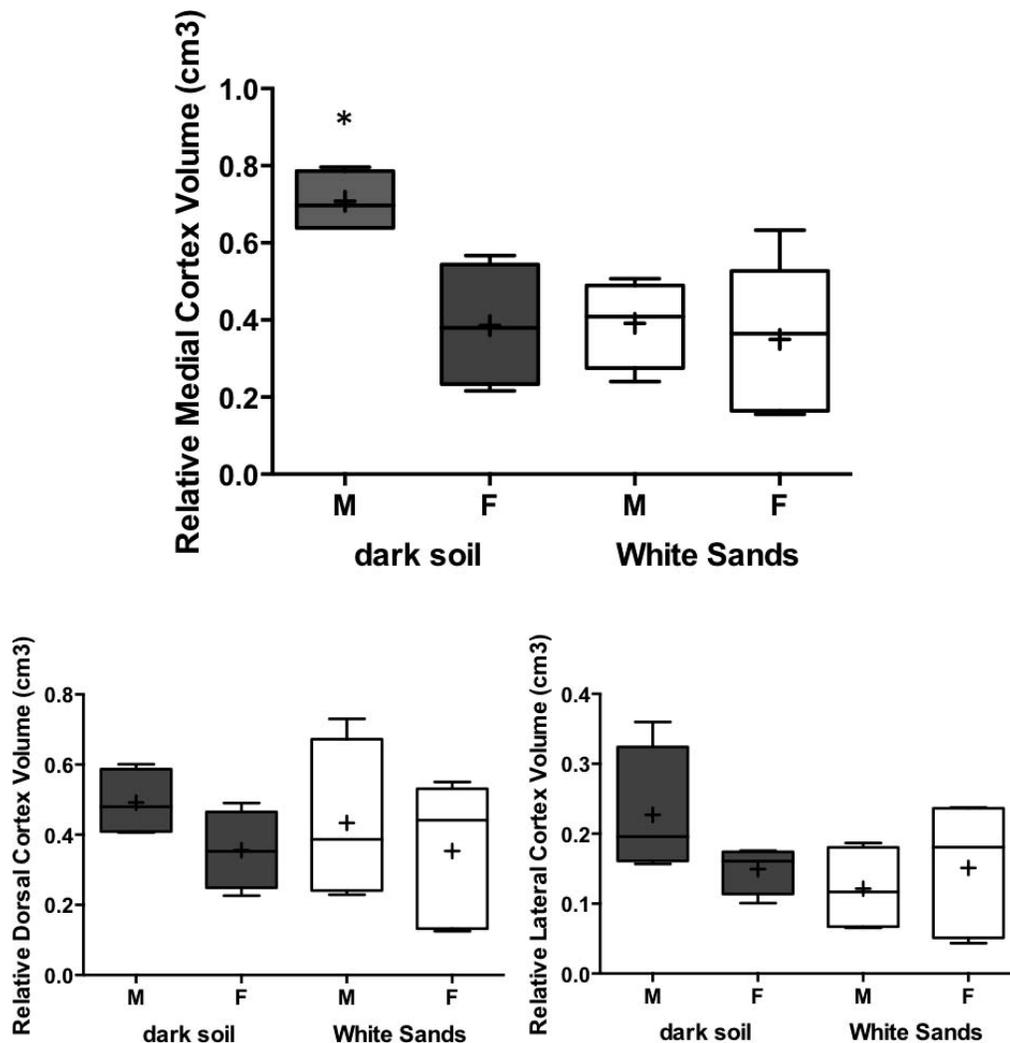


FIG. 3. Box and whisker plots depicting the relative size of the medial (top), dorsal, and lateral (bottom) cortices, for male and female *H. maculata* from the dark soil and White Sands populations. An asterisk denotes a significant difference between means ( $\alpha = 0.05$ ), + symbols signify the means, the midlines are the medians, box ends are the 25th and 75th percentiles, and whiskers are the maximum and minimum values.

One hypothesis regarding the mechanism underlying the observed neuroanatomical differences between the lizards in the different habitats is that natural selection may be acting on the medial cortex to increase fitness for the individuals living in the ancestral environment. Comparative studies in lizards, birds, and mammals have reported that the size of the homologous structure of the hippocampus is affected by selective pressures, to the degree by which the individual relies on spatial information to locate and utilize resources (e.g., Sherry et al., 1992; Powell and Leal, 2012; Roth et al., 2012). Assuming that there is a cost to maintaining neural substrates (Jerison, 1975), selection should not favor the development and maintenance of a larger than necessary brain structure. For example, if White Sands *H. maculata* (particularly males) have a reduced need for navigational abilities or spatial processing in their seemingly less complex environment, the size of the medial cortex may diminish over an individual's lifespan, or through evolutionary time. Alternatively, growth in this region, resulting from postnatal neurogenesis, may occur to a lesser degree than in the ancestral habitat. In either event, the question remains as to how *H. maculata* actually perceive habitat complexity and whether differences observed in the medial cortex volume are heritable and have direct consequences on fitness.

In contrast to the strong patterns observed for the medial cortex, we did not find significant population or sex differences in the dorsal and lateral cortex volumes. The dorsal cortex, homologous to regions of the mammalian hippocampus, has been associated with differences in the use of space (Day, 1999; LaDage et al., 2009), whereas the lateral cortex has been found to be homologous to the mammalian olfactory cortex (Martinez-Garcia et al., 1986). Although we found no significant results for either of these brain regions, we observed a trend for males from the dark soil habitat to have larger relative lateral cortex volumes, compared to the other groups (Fig. 3, medium effect size). Further work will be necessary to understand whether the environment truly affects the dorsal and lateral cortices in this system.

In summary, we identified a volumetric difference of the medial cortex in lizards associated with a recent environmental shift. We also identified differences in this neuroanatomical structure between the sexes. In a wild population of animals, we offer support for the hypothesis that the environment—and the way that organisms are influenced by their environment—can shape neuroarchitecture. Understanding the specific neural mechanisms responsible for observed differences in medial cortex volume could be gained by incorporating additional

metrics, such as neuron size and abundance and variation in cell connectivity (Roth et al., 2010). Understanding the ecological, evolutionary, and developmental mechanisms responsible for observed patterns also requires further work. We have not yet formally evaluated the relative contribution of adaptive genetic change, genetic drift, and developmental plasticity to the observed neuroanatomical changes, and common garden breeding experiments would be an important next step; however, our data demonstrate the importance of studying the brain's interaction with rapidly changing environments. Our findings also emphasize the potential that sex-specific neuroanatomical patterns may be more widespread in vertebrates than currently appreciated; therefore, evaluating males and females simultaneously, something that has been rarely done in neurobiological studies, is important (Zucker and Beery, 2010; Beery and Zucker, 2011; Clayton and Collins, 2014).

*Acknowledgments.*—We thank White Sands National Monument, White Sands Missile Range, Jornada Long-Term Ecological Research Station, and the New Mexico Department of Game and Fish for providing permits and access to field sites. We thank T. Roth for his insightful discussion of this work, and the Rosenblum Lab, especially K. Hardwick, for helping collect lizards and discussing this project. We also thank S. Des Roches, who kindly granted us permission to use her images for Figure 1, and S. Diaz-Muñoz for the Spanish translation of our abstract. All use of vertebrate animals for this study was approved by the White Sands National Monument, White Sands Missile Range, Jornada Long-Term Ecological Research Station, the New Mexico Department of Game and Fish (3184), and the University of California Animal Care and Use Committee (ACUP R347). Funding was provided by the National Science Foundation (DEB-1054062 to EBR) and by the University of California's President's Postdoctoral Fellowship (to RMC).

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Accepted: 3 January 2017.

Published online: 3 April 2017.