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The effect of environmental harshness on neurogenesis: a large-scale comparison

Leia V. Chancellor¹, Timothy C. Roth¹, Lara D. LaDage¹, and Vladimir V. Pravosudov^{1,*}

¹ Department of Biology, University of Nevada, Reno, NV 89557, USA

Abstract

Harsh environmental conditions may produce strong selection pressure on traits, such as memory, that may enhance fitness. Enhanced memory may be crucial for survival in animals that use memory to find food and, thus, particularly important in environments where food sources may be unpredictable. For example, animals that cache and later retrieve their food may exhibit enhanced spatial memory in harsh environments compared to those in mild environments. One way that selection may enhance memory is via the hippocampus, a brain region involved in spatial memory. In a previous study, we established a positive relationship between environmental severity and hippocampal morphology in food-caching black-capped chickadees (*Poecile atricapillus*). Here, we expanded upon this previous work to investigate the relationship between environmental harshness and neurogenesis, a process that may support hippocampal cytoarchitecture. We report a significant and positive relationship between the degree of environmental harshness across several populations over a large geographic area and 1) the total number of immature hippocampal neurons, 2) the number of immature neurons relative to the hippocampal volume, and 3) the number of immature neurons relative to the total number of hippocampal neurons. Our results suggest that hippocampal neurogenesis may play an important role in environments where increased reliance on memory for cache recovery is critical.

Keywords

black-capped chickadee; food hoarding; latitudinal gradient; hippocampus; neuron; spatial memory

INTRODUCTION

Memory is an important mechanism that allows organisms to recall features of their environment that may be critical for survival. There appears to be variation in memory function among species and such variation may be explained in part by the role that memory plays in fitness (Sherry, 2006; Shettleworth, 2003). Although some of the differences in memory among species are likely the result of differing selection pressures (Krebs et al., 1989; Sherry and Schacter, 1987; Sherry et al., 1989), the mechanisms through which selection might affect memory and the exact role of selection on memory remains poorly understood.

One way that selection may affect memory is via the hippocampus (or hippocampal formation in birds; Hp), a brain region involved in memory function. The role of the Hp in spatial memory has been well documented both in birds and mammals (Bast, 2007;

*Direct all correspondence to: Vladimir V. Pravosudov Department of Biology, MS 314 University of Nevada Reno Reno, NV 89557 vpravosu@unr.edu.

Shettleworth, 1998), and there seems to be a great deal of variation in the morphology of this region across species (e.g. Sherry, 2006). This variation in morphology tends to be related to spatial memory use, in that increased reliance on spatial memory is associated with increased Hp attributes (Krebs et al. 1989; Sherry, 2006; Sherry et al., 1989).

Spatial memory and the Hp appear to be particularly important for animals that cache and later retrieve their food (Krebs et al., 1989; Sherry et al., 1989; Shettleworth, 1995; Vander Wall, 1990). Food-caching species tend to have larger Hp formations compared to non-caching species (Clayton, 1995; Krebs et al., 1989; Sherry et al., 1989; but see Roth et al. 2010). Also, damage to the Hp can inhibit the accuracy of spatially-based cache retrieval, although it does not interfere with other aspects of memory, such as memory for color, or food caching behavior in general (Hampton & Shettleworth, 1996; Sherry & Vaccarino, 1989). This suggests that the Hp in food caching species may be specialized to a degree for some aspect of spatial memory involved in cache retrieval.

In addition to variation across species, there is variation in Hp morphology within species. One possible source of variation in the Hp is differential reliance on spatial memory as a result of environmental severity. The effect of environmental conditions on memory may be particularly relevant for non-migratory species of food-caching birds, as they lack predictable food sources during the winter months and compensate by relying heavily upon cached food (Krebs et al., 1989; Pravosudov & Clayton, 2002; Pravosudov & Lucas, 2001; Sherry et al., 1989). In harsh environments characterized by low ambient temperature and heavy snowfall, food-caching birds must spend a significant portion of the day locating and consuming cached food to meet increased metabolic requirements (Hurly, 1992; McNamara et al., 1990; Pravosudov & Grubb, 1997a, 1997b). In addition, birds living in northern climates have another limitation. Due to shorter day lengths during the winter, especially at higher latitudes, the time available in which to accumulate the larger requisite energy reserves sufficient for overnight survival is greatly reduced. . Thus, the ability to retrieve cached food items is more likely to be important in these more northern environments. Thus, more caches and more accurate cache retrieval may be necessary in more harsh environments, especially at higher latitudes during the winter. Because the retrieval of caches relies in part on spatial memory, and spatial memory is facilitated in part by the Hp, selection may produce enhanced spatial memory and increased Hp attributes in populations living in harsh environments in spite of some potential physiological costs of maintaining a larger hippocampus (Pravosudov & Grubb, 1997b; Pravosudov & Lucas, 2001). For example, Pravosudov & Clayton (2002) showed that black-capped chickadees (*Poecile atricapillus*) from a harsh Alaskan environment had significantly larger Hp regions with more neurons and performed better on spatial memory-based tasks compared to chickadees from a milder Colorado environment. In a follow-up study, Roth & Pravosudov (2009) confirmed this pattern on an extended environmental transect that included multiple populations across the species' range.

There is some debate on whether some caching species use memory for the retrieval of long-term caches, but it appears undisputable that chickadees use memory for short-term retrieval (Brodin, 2005a). Even if chickadees indeed use spatial memory only on a short-term basis, selection for better memory should still be more intense in more harsh environments, where daily survival may still be highly dependent upon successful cache retrieval, even of short-term caches and re-cached food (e.g. Pravosudov & Lucas, 2001). Failure to find previously made caches even on a short temporal scale may have severe survival consequences in harsh environments.

Our previous study (Roth & Pravosudov, 2009), however, did not examine the pattern of neurogenesis, which may also be associated with environmental harshness. The addition of

new neurons in the Hp may be important in processing new spatial memories of cache locations (Barnea & Nottebohm, 1994). Since spatial memory is important for retrieving food caches, neurogenesis in the Hp may be one mechanism through which natural selection may have a pronounced effect on memory. Here, we examined the expression of doublecortin (DCX; a measure of neurogenesis) in Hp neurons in black-capped chickadees along an environmental gradient using the same individuals from the same five populations as reported in Roth and Pravosudov (2009). We hypothesized that neurogenesis is related to environmental harshness and that a positive relationship exists between the severity of the environment and the number of immature neurons on a latitudinal gradient of environmental harshness across North America.

METHODS

Collection Sites & Chickadee Capture

The sampling locations (Manhattan, KS; Fort Collins, CO; Missoula, MT; Prince George, BC; Fairbanks, AK) were selected based on their average winter snowfall, temperature, and day length, creating an environmental scale ranging from mild to severe (see Roth & Pravosudov, 2009 for specific data on these environmental variables). Twelve chickadees were captured from feeders established in each location as part of the original study (Roth & Pravosudov, 2009). All birds were captured during their predicted peak in caching, from mid-September in Alaska to late October in Kansas (Brodin, 2005b; Pravosudov et al., 2006; see Roth & Pravosudov, 2009). Tissue from 10 birds from Alaska, 6 birds from British Columbia, 7 birds from Montana, 8 birds from Colorado, and 7 birds from Kansas were selected from each population for the analysis. Sample sizes are unequal due to variation in the quality of staining. Only tissue that was adequately stained and could be reliably measured was used in the analysis. Staining quality was determined blind to location and individual identity by TCR.

Chickadee Brain Tissue Preparation

As described in Roth and Pravosudov (2009), the chickadees were euthanized, sexed via gonadal inspection, and their brains extracted shortly after capture. Briefly, birds were administered an overdose (0.07 ml of 50 mg/ml) of sodium pentobarbital and then transcardially perfused with 0.1 M phosphate-buffered saline, then methanol free 10% formalin (from paraformaldehyde). The brains were immediately extracted from the skull, post-fixed in methanol free 10% formalin (from paraformaldehyde) for one week, cryoprotected with 15% and 30% sucrose solutions, and then stored at -80°C . The brains were sectioned coronally (40 μm thick) on a Leica CM 3050S cryostat at -20°C and frozen in a cryoprotectant composed of ethylene glycol and glycerol (5: 6) until analysis.

DCX Immunohistochemistry

Immunohistochemistry was used to visualize the doublecortin protein, which is associated with neuronal cell microtubule machinery localized in newly developing neurons (Balthazart et al., 2008; Gleeson, 1999). Although many previous studies typically use BrdU (5-bromo-2-deoxyuridine) to visualize neurogenesis (see Taupin, 2007 for examples), we could not do so for logistical reasons. BrdU is useful in situations where multiple injections can be administered. However, in this study, BrdU administration was not logistically feasible since the same wild birds would have to have been captured, injected with BrdU, released, and then recaptured. To circumvent this, we used DCX, an endogenous marker of developing neurons. In passerine birds, DCX expression occurs for 25–30 days after the production of a new neuron, after which point expression of the protein ceases (Balthazart et al., 2008). Following this developmental stage, neurons begin to express NeuN, a marker of mature neurons (Mullen et al., 1992). Consequently, DCX appears to be a relevant marker for

young, developing neurons (Brown et al. 2003). Previous studies have also demonstrated via direct comparison that DCX and BrdU labeling provide similar results following standard exercise protocol (e.g. Couillard-Despres et al., 2005). Overall, there seems to be ample evidence suggesting that DCX is a comparable technique to estimate neurogenesis. However, DCX addresses a slightly different aspect of neurogenesis than BrdU, as it does not allow for the investigation of neuronal survival by providing a combined estimate for neuron production and survival. Thus, our conclusions drawn from DCX are relevant only as an estimate of the population of neurons remaining in the brain that were produced within 25–30 days of sacrifice. For a full description of the benefits and limitations of DCX as a measure of neurogenesis, see LaDage et al. (2010).

We followed the DCX-staining protocol optimized for chickadees by LaDage et al. (2010). Briefly, we washed the tissue sections in Tris-buffered saline (TBS), and then incubated them in 30% hydrogen peroxide and TBS (1: 50.0) for 30 min at room temperature. The sections were then washed in TBS and incubated at room temperature for 30 min in blocking buffer consisting of normal horse serum (1: 33.3), TX-100 (1: 39.8) and TBS. Following this, the sections were incubated overnight for approximately 18 h at 4°C in anti-doublecortin antibody made in goat and blocking buffer (1: 200; Santa Cruz Biotechnology, Santa Cruz, CA, SC-8066). On the second day of the staining process, the tissue sections were washed in TBS and then incubated at room temperature for 2 h in biotinylated horse anti-goat antibody in blocking buffer (1: 200, Vector Laboratories, Burlingame, CA, US, BA-9500). Next, the tissue sections were again washed in TBS and incubated at room temperature for one h in ABC Elite kit (Vector Laboratories, PK-6100). After this, the tissue sections were reacted with DAB + nickel kit (Vector Laboratories, SK-4100) for 2 min at room temperature. The slides were then washed one last time in TBS and mounted onto slides. The slides were dried at 37 °C overnight and then lightly Nissl stained to ascertain the lateral Hp boundaries.

Chickadee Brain Analysis

Boundaries for the Hp (specifically the hippocampal formation, the hippocampus and parahippocampus regions combined) were determined as per our previous studies (LaDage et al., 2010; Pravosudov & Clayton, 2002; Roth & Pravosudov, 2009). The number of DCX-positive cells present in the Hp was estimated on every twelfth section using the optical fractionator technique (West et al., 1991) and followed our previous studies (Fox et al., 2010; LaDage et al., 2010;). Neurons were counted using the StereoInvestigator software (MicroBrightField, Inc., Colchester, VT, USA) on a Leica M4000B Light Microscope (Bannockburn, IL, USA).

We used a counting frame of 70 × 70 µm on a 250 µm grid with a fixed dissector height of 5 µm at 1000X (LaDage et al., 2010). DCX-stained neurons (e.g., Figure 1) were identified by their dark brown color, which contrasted with the lightly blue Nissl-stained (non-DCX-expressing) neurons. All tissues were analyzed blind to location and individual identity by LVC.

Statistical Analysis

To test the prediction that the number of immature neurons in chickadees increases along a gradient of environmental harshness, data were analyzed using an ordered heterogeneity test to evaluate the relationship between environmental harshness and neurogenesis (Gaines & Rice, 1990; Rice and Gaines, 1994; see also Roth & Pravosudov, 2009). This test has been specifically designed to test directional trends across 3 or more groups and uses aspects of the General Linear Model and Spearman's rank correlation coefficient to calculate its test statistic (see Gaines & Rice, 1990; Rice & Gaines, 1994). Our main test focuses on the

relationship between the absolute number of DCX-labeled cells across the latitudinal gradient. However, we have previously shown differences in Hp volume and Hp neurons along this gradient (Roth & Pravosudov, 2009). Northern birds tend to have larger hippocampal volumes with more neurons (Roth & Pravosudov, 2009). Consequently, we have included two additional analyses to test whether variation in neurogenesis due to location was independent of the differences in either the hippocampal volume (in effect, density) or the total number of hippocampal neurons (in effect, proportion) by using these variables as covariates. Using hippocampal volume and total neuron number as covariates in multivariate General Linear Models has been suggested to be a preferable statistical procedure to ratios (e.g. number of neurons/hippocampal volume, number of DCX-positive neurons/total number of neurons) when testing for the relative effects of these variables, as using ratios may produce unreliable and biased results (e.g., Blem, 1984; Raubenheimer, 1995; Jasienski & Bazzaz, 1999; Packard & Boardman 1999).

Traditionally, telencephalon/brain volume and/or body mass have been used as control areas in the studies of the hippocampal volume (Krebs et al., 1989; Sherry et al., 1989). However, there appears to be no reason to think that neurogenesis should be related to either telencephalon volume or body mass. In addition, selection pressures may produce independent changes both in telencephalon and body mass in different populations, in which case using these variables as controls for neurogenesis might produce misleading results. Therefore, we did not control for telencephalon volume or body mass, but instead controlled for the total number of hippocampal neurons and hippocampal volume, which may potentially be related to neurogenesis rates (LaDage et al., 2010). We note for heuristic value that even when these covariates are included, the relationships remain the same.

There was no significant difference in the number of DCX-positive cells in the Hp between the sexes ($F_{1,32} = 1.08$, $p = 0.306$), so data for the sexes were pooled.

RESULTS

Significant differences in the number of DCX-positive neurons were found between the right and left Hp hemispheres. The right hemisphere possessed approximately 15% more DCX-positive Hp neurons than the left hemisphere (repeated measures ANOVA, $F_{1,33} = 34.2$, $p < 0.001$). There was a significant location effect ($F_{4,33} = 3.55$, $p = 0.016$), but no significant interaction between location and hemispheric side-biased DCX expression ($F_{4,33} = 0.71$, $p = 0.59$), so the left and right hemispheres were combined for subsequent analyses.

The total number of DCX-positive neurons differed significantly across locations ($r_sP_c = 0.984$, $k = 5$, $p < 0.001$), aligning on a gradient of environmental severity. Locations with more harsh environments expressed significantly more DCX-positive neurons (Fig1). The number of DCX-labeled neurons relative to the hippocampal volume (in effect, “density”, i.e., when hippocampal volume was used as a covariate) also followed this trend ($r_sP_c = 0.588$, $k = 5$, $p < 0.05$). Finally, the number of DCX-labeled neurons relative to the total number of all hippocampal neurons (in effect, “proportion”, i.e., when the total number of all neurons was used as a covariate) also showed a significant and expected trend along a gradient of environmental severity ($r_sP_c = 0.98$, $k = 5$, $p < 0.001$).

DISCUSSION

We found a significant and positive relationship between environmental harshness and Hp neurogenesis as measured by the number of DCX-stained neurons in black-capped chickadees. In addition, the number of DCX-labeled neurons showed a significant trend along the environmental gradient independent of the already shown differences in the

hippocampal volume and the total number of hippocampal neurons (Roth & Pravosudov, 2009). A final, unexpected finding was that, in all populations, the right Hp contained significantly more DCX-stained neurons than the left.

The observed pattern of neurogenesis in relation to environmental conditions supports our main hypothesis that Hp neurogenesis is related to environmental severity. Combined with our previous findings (Roth and Pravosudov, 2009), our data suggest that birds in harsher environments have larger Hp with more neurons and relatively more immature neurons, all of which may support enhanced spatial memory (Pravosudov & Clayton, 2002; Pravosudov & Grubb, 1997b; Pravosudov & Lucas, 2001; Roth & Pravosudov, 2009). The increase in the number of immature neurons along the environmental gradient was independent of the similar increase in the total number of hippocampal neurons (Roth & Pravosudov, 2009), suggesting that northern chickadees had more new neurons relative to their total number of neurons. Neurogenesis may be a possible mechanism for providing support for neural architecture in the Hp and may in part support the larger population of mature neurons found in our previous study (Roth & Pravosudov, 2009). An alternative explanation is that the increased number of DCX-positive neurons reflects a higher rate of neurogenesis due to a faster neuronal turnover rate in birds from more harsh environments (Barnea & Nottebohm, 1994). Regardless, since the addition of new neurons in the Hp may be important in processing spatial memories of cache locations (Barnea & Nottebohm, 1994), it is possible that neurogenesis in the Hp may enhance spatial memory and, thus, facilitate survival in harsh conditions.

We also found significantly more DXC-labeled neurons in the right hemisphere of the Hp than in the left, and this relationship was consistent across all populations analyzed. There were no significant differences between hemispheres in the total number of Hp neurons in these birds (Roth & Pravosudov, 2009), yet the number of immature neurons was larger specifically in the right Hp. Such a pattern suggests that neuronal turnover rate may be especially high in the right Hp. Interestingly, the hemispheric differences in neurogenesis found in this study have not been noted in previous studies with chickadees (e.g., Fox et al., 2010; LaDage et al., 2010; Tarr et al., 2009), although lateral Hp differences have been noted in other bird species (e.g., Gagliardo, 2001; Pravosudov et al., 2006). Previous studies have suggested that the right Hp hemisphere may be involved in the processing of global spatial memory, while the left hemisphere has been implicated in the processing of spatial memory on a more local scale (Kahn & Bingman, 2004). We speculate that this right-hemisphere bias may represent differences in the processing of environmental information. The right hemisphere has been suggested to be specifically involved in processing of global spatial information about the environment, which may be crucial for remembering cache locations within a home range. In contrast, the left hemisphere may deal primarily with local cues (Kahn & Bingman, 2004), which may be less reliable due to seasonal changes in the environment. Spatial memory for global landmarks may also be particularly important when local landmarks are modified by severe weather conditions. It is interesting to note that our birds were collected during early autumn during the peak of their long-term caching when demands for memory encoding may be highest. Further research is needed to uncover why this may be and how to interpret the significance of such lateralization.

Neurogenesis was only measured in the Hp in this study. Consequently, we cannot conclude that the pattern found is only relevant to the hippocampal neurogenesis, as our results may reflect larger scale neurogenesis in the entire brain. Nevertheless, the pattern of hippocampal DCX-expression across the environmental gradient is significant in terms of raw numbers of immature neurons as well as relative to the hippocampal volume and the total number of hippocampal neurons. This pattern is consistent with our previous observations and expectations. However, given the complexity of the brain and its role in the expression of

numerous, potentially adaptive, and complex behaviors (e.g., other aspects of cognition such as learning), we suggest that it may be naïve to speculate that the Hp alone would be the only brain structure subject to selection pressures, such that another arbitrary region of the brain could act as a baseline measure of neurogenesis. Indeed, using some of the same populations, we have found large differences in problem-solving and neophobia between birds at the extremes of the environmental gradient (Roth et al., 2010b). These behavioral differences very likely parallel morphological differences in regions of the brain other than the Hp such as the nucleus taeniae amygdalae, area corticoidea dorsolateralis, caudolateral nidopallium, and the hyperpallium apicale (see Burns et al., 1996; Hartmann & Güntürkün, 1998; Kröner & Güntürkün, 1999; Quilodran et al., 2008). Since we expect differences in these and other regions, it is difficult and perhaps incorrect to arbitrarily measure neurogenesis in other randomly selected regions, to assume that these other regions may reflect global neurogenesis, and to assume that such measurements in some way may serve as a control for our investigation of neurogenesis in the Hp. It has been suggested that, when all else is equal, increases in neurogenesis within individuals or treatment groups are associated with enhanced memory performance (Aimone et al. 2010). Thus, within our intraspecific comparison, it may be likely that increased neurogenesis in the hippocampus may affect memory irrespective of whether other parts of the brain also experience changes in neurogenesis, and such changes in memory may have an impact on fitness in food-caching chickadees.

While we argue that selection may have produced the observed increase in neurogenesis in more harsh locations as a means to improve survival via more successful cache recovery, it is also possible that the differences in neurogenesis among populations may be simply due to differences in memory use. For example, we have established that restricting spatial memory-based tasks reduces neurogenesis in birds (LaDage et al., 2010). This has also been demonstrated in mammals, where the process of learning a spatial memory-based task has been shown to have stage-specific effects on neurogenesis (Döbrössy et al., 2003). These types of studies indicate that memory exercise can directly modulate neurogenesis. Thus, the birds in more harsh environments may cache more food (Pravosudov and Clayton, 2002) and consequently may use their memory more extensively compared to their southern conspecifics. This could, in turn, directly cause the observed differences in neurogenesis. Whether differences in neurogenesis between these populations are a product of selection or produced by memory-related exercise merits further study.

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Figure 1.
A representative example of a DCX-stained neuron (dark brown) at 1000x magnification.

Figure 2.
Mean number of DCX-stained Hp neurons across a gradient of environmental harshness.
Error bars are SE.