

University of Nevada, Reno

**Climate-Related Variation in Spatial Memory and Hippocampal Morphology in
Food-Caching Chickadees.**

A thesis submitted in partial fulfillment of the
requirements for the degree of Master of Science in

Biology

By

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Abstract

Harsh environments may lead to increased demands on memory in animals that rely on memory for survival. We previously showed that winter severity is associated with non-experience-based differences in memory and the hippocampus over a large continental scale in food-caching black-capped chickadees (*Poecile atricapillus*). However, large climatic differences also occur along steep elevational gradients in montane environments over a small geographic scale. Here we demonstrate for the first time that large differences in memory and the hippocampus exist over extremely short distances (10km) along the elevation gradient. We discovered that food-caching mountain chickadees (*P. gambeli*) from the highest elevations in the Sierra Nevada Mountains exhibited significantly better spatial memory associated with larger hippocampi with almost twice the number of hippocampal neurons compared to individuals only 600m lower in elevation. We found similarly large differences in hippocampal neurogenesis rates as indicated by the total number of immature neurons. Our study therefore suggests that climate-related environmental differences can produce dramatic differences in memory and the hippocampus in animals within close proximity on small spatial scales and that currently observed trends in global climate may have significant effects on cognition and the brain.

Additionally, we attempted to integrate a new metric for enhanced spatial memory by looking specifically at the morphology of the neuron. While most comparative studies of cognition have focused on volumetric brain measurements it remains unclear whether neuron morphology, which appears to be directly linked to cognitive functions, may be

responsive to differential selection on cognitive ability. We show that neuron soma size in the hippocampus, exhibits significant population variation associated with different environmental pressures on spatial memory related to differences in winter climate harshness in two species of food-caching chickadees. Comparing ten populations of black-capped chickadees and three populations of mountain chickadees along a gradient of winter climate harshness, we found that birds from harsher environments had significantly larger hippocampal neuron soma sizes. Finally, using chickadees from the two most divergent populations reared in a laboratory environment, we showed that these differences appear to be at least partly heritable as significant differences between these populations remained in birds sharing the same laboratory environment. At the same time, laboratory reared birds had significantly smaller neuron soma size compared to the wild-sampled birds, suggesting that at least some variation in neuron soma size may be due to environment-related plasticity. Our data suggests that environment-related selection on memory may generate differences in neuron morphology, which appear to be controlled by some heritable mechanisms and likely underlie population differences in spatial memory.

All multi-media elements in this thesis are in JPEG (.jpeg) format.

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Chapter 1

Introduction

Cognitive traits such as learning and memory are important for animals living in unpredictable environments. Since enhanced cognitive traits allow animals to respond to environmental change, high degrees of environmental complexity and unpredictability are hypothesized to result in the evolution of these traits and their underlying neural infrastructure (Krebs et al. 1989, Sherry et al. 1989, 1992, Barton & Harvey 2000, Pollen et al. 2007, Pravosudov & Smulders 2010). Selection for enhanced cognitive traits, such as spatial memory, should be especially high in environments where they are critical for survival. In the context of spatial memory, strong environmental pressures should affect the hippocampus, a brain structure involved in spatial memory (Krebs et al. 1989, Sherry et al. 1989, 1992, Pravosudov & Smulders 2010). Spatial memory in food-caching animals should be critical for survival and thus provide strong selection for enhanced cognitive traits since food-caching animals rely, at least partially, on memory, to recover previously cached food in order to survive the winter (Krebs et al. 1989, Sherry et al. 1989, 1992, Pravosudov & Smulders 2010). This caching behavior is thought to have evolved in order to provide a predictable food source during periods of environmental instability and seasonal change resulting in periods of resource scarcity (Vander Wall 1990). Food-caching and enhanced spatial memory in small non-migratory birds should be especially valuable in environments that experience harsh winter climates. In these harsh environments food-caching birds would benefit from better spatial memory in order to retrieve caches and as a result environmental harshness may drive selection for

enhanced spatial memory and the underlying hippocampal morphology (Pravosudov & Clayton 2002, Roth & Pravosudov 2009, Roth et al. 2011, 2012).

The food-caching paradigm is a wonderful model for studying cognition. There are numerous species of passerine bird species that retrieve previously cached food items. These include species of nuthatches, jays, crows, chickadees, and tits (Sittidae, Corvidae, and Paridae families). Individuals place a small number of food items in many separate cache locations throughout the home range (Sherry 2006). The scattered distribution of these caches is believed to be an anti-pilfering behavioral strategy. Caching usually occurs in non-migratory bird species during the fall for retrieval over the winter months. Thus caches provide a reliable food resource during the winter months when A.) there are increased metabolic demands due to lower ambient temperatures and B.) increased food scarcity common during winter months. Once winter arrives birds need the ability to accurately retrieve these caches. Multiple experimental studies have shown that birds retrieve their caches through the use of memory (Sherry et al. 1989, Shettleworth & Krebs 1982).

The area of the brain associated with spatial memory is the hippocampus (Krebs et al. 1989). When the hippocampus is experimentally lesioned in Black-capped Chickadees, *Poecile atricapillus*, cache retrieval is disrupted (Sherry & Vaccarino 1989). There is also a strong relationship between the size of the hippocampal region and caching behavior, when comparing food-caching species and non food-caching species. Food-caching species have larger hippocampal volumes relative to body size compared to non food-caching species (Krebs et al. 1989, Sherry et al. 1989). These studies look specifically at

the spatial memory demands and hippocampal morphology between species, but it is also possible to do comparative studies at an intraspecific level by comparing populations inhabiting different climates. By focusing on a single species and sampling multiple populations, one is able to remove the possibility of ecological confounds and the phylogenetic assumptions present in interspecific comparative studies (Roth and Pravosudov 2009). Environmental harshness can be used as a metric to study spatial memory demands in food-caching species that inhabit a large range of climates. These harsh conditions would be characterized by longer periods of lower ambient temperatures, longer periods of snowfall, and prolonged periods of snow cover (Pravosudov & Clayton 2002). Food-caching non-migratory populations living in these environments should experience increased metabolic demands and longer periods of food scarcity. These high metabolic demands coupled with increased food scarcity should increase reliance on previously cached food reserves. Since food-caching animals use memory to retrieve caches, under more harsh winter conditions there should be strong selection for enhanced spatial memory. Increased demands for enhanced spatial memory would be met with a robust neural infrastructure in the hippocampus.

Previous studies have shown a strong relationship between harsh winter climates and enhanced spatial memory and the corresponding neural infrastructure in the hippocampus across a large continental scale both along latitudinal and longitudinal gradients in Black-capped Chickadees (Pravosudov & Clayton 2002, Roth & Pravosudov 2009, Roth et al. 2011, Chancellor et al. 2011). Individuals from populations that experience a more harsh winter climate exhibit better spatial memory, have larger hippocampi with more hippocampal neurons, and higher levels of hippocampal

neurogenesis (Pravosudov & Clayton 2002, Roth & Pravosudov 2009, Roth et al. 2011, Chancellor et al. 2011). An additional study using lab-raised individuals from the most divergent populations suggests that the observed differences in hippocampal morphology may be inherited and not based on differences in memory-based experiences (Roth et al. 2012).

In this work we explore whether the large differences in environmental harshness can produce these same variations in spatial memory and hippocampal morphology along a small spatial scale. Large differences in environmental harshness are evident along the elevational scale in a montane environment, similar to those observed across our previous large scale studies. Higher elevations experience lower ambient temperatures, longer periods of snow fall, and longer periods of snow cover when compared to lower elevations. We hypothesized that the harsh climates at high elevations will increase demands and select for enhanced spatial memory and hippocampal morphology in food-caching Mountain Chickadees (*Poecile gambeli*). We predicted that Mountain Chickadees living at higher elevations would exhibit better spatial memory, larger hippocampal volumes, more hippocampal neurons, and higher rates of hippocampal neurogenesis when compared to individuals living at lower elevations.

Additionally, we investigate the possible mechanism underlying observed variation in hippocampal volume. Most comparative studies involving cognition and spatial memory have focused on volumetric brain measurements as an indication of enhanced memory. What has been previously unexplored is the role of neuron morphology, specifically neuron soma size, in the maintenance of observed variation in

the volume of these brain regions and if the neuron morphology is similarly responsive to selection on cognitive ability. We measured hippocampal neuron soma sizes in the ten populations of Black-capped Chickadees along the continental scale of climate harshness, and the three populations of Mountain Chickadees along the elevational gradient representing climate harshness. We hypothesized that hippocampal neuron soma size would be positively associated with increased environmental harshness, resulting in the largest hippocampal neuron soma sizes being found in individuals living in the most harsh climates. Finally, we measured hippocampal neuron soma sizes in the two lab-raised populations of Black-capped Chickadees representing the most divergent climates to discover if the observed variations in neuron soma sizes were plastic or fixed and thus mediated by some heritable mechanisms.

Chapter 2

Title: Elevation-Related Differences in Memory and the Hippocampus in Mountain Chickadees (*Poecile gambeli*)

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Abstract

Harsh environments may lead to increased demands on memory in animals that rely on memory for survival. We previously showed that winter severity is associated with non-experience-based differences in memory and the hippocampus over a large continental scale in food-caching black-capped chickadees (*Poecile atricapillus*). However, large climatic differences also occur along steep elevational gradients in montane environments over a small geographic scale. Here we demonstrate for the first time that large differences in memory and the hippocampus exist over extremely short distances (10km) along the elevation gradient. We discovered that food-caching mountain chickadees (*P. gambeli*) from the highest elevations in the Sierra Nevada Mountains exhibited significantly better spatial memory associated with larger hippocampi with almost twice the number of hippocampal neurons compared to individuals only 600m lower in

elevation. We found similarly large differences in hippocampal neurogenesis rates as indicated by the total number of immature neurons. Our study therefore suggests that climate-related environmental differences can produce dramatic differences in memory and the hippocampus in animals within close proximity on small spatial scales and that currently observed trends in global climate may have significant effects on cognition and the brain.

Keywords: cognition, memory, hippocampus, food caching, chickadees, climate

Introduction

Unpredictability and complexity of the environment have been hypothesized to result in the evolution of enhanced cognition and associated neural circuits (Krebs et al. 1989, Sherry et al. 1989, 1992, Barton & Harvey 2000, Pollen et al. 2007, Pravosudov & Smulders 2010). These specific selection pressures may be expected to affect specific neural circuits within the brain (Krebs et al. 1989, Sherry et al. 1989, 1992, Barton & Harvey 2000, Pollen et al. 2007, Pravosudov & Smulders 2010). Consequently, environments in which spatial memory may be critical for survival may provide especially strong selection pressure for enhanced memory and the hippocampus, an area of the brain involved in memory function (Krebs et al. 1989, Sherry et al. 1989, 1992, Pravosudov & Smulders 2010). Food-caching animals present a great model to investigate the relationship between environment, memory and the hippocampus because they rely on food caches to survive winter and they depend, at least in part, on memory, to relocate previously made caches (Krebs et al. 1989, Sherry et al. 1989, 1992, Pravosudov & Smulders 2010). It is widely assumed that food caching has evolved in

response to seasonally changing environments so that food-caching animals may provide a predictable food supply during periods of seasonal food scarcity (Vander Wall 1990). In addition, many animals may engage in short-term caching to ensure that they can obtain enough food to survive the night or short periods during inclement weather (Pravosudov & Grubb 1997). We hypothesized that in many small birds food caching would be especially valuable in harsh winter climates characterized by low ambient temperatures, which usually increase metabolic requirements, and by snowfall and snow cover, which may restrict access to naturally available food (Pravosudov & Clayton 2002). As a result, it may be generally expected that aside from any other potential physiological adaptations to cold climate, small food-caching birds in more harsh environments might gain more benefits from enhanced memory, which is needed to successfully recover their caches (Pravosudov & Clayton 2002, Roth & Pravosudov 2009, Roth et al. 2011, 2012). Climate-related environmental harshness therefore may be one of the main driving forces behind the evolution of enhanced memory and hippocampal structure, at least in small-bodied food-caching avian species.

We have previously shown that, over a large continental scale along both longitudinal and latitudinal climate gradients, a more severe winter climate is associated with enhanced spatial memory, an enlarged hippocampus with more neurons, and more intense neurogenesis in multiple populations in a small songbird species - the non-migratory food-caching black-capped chickadee, *P. atricapillus*, (Pravosudov & Clayton 2002, Roth & Pravosudov 2009, Roth et al. 2011, Chancellor et al. 2011). Moreover, we showed that such differences in memory and the brain cannot be explained simply by differences in memory-based experiences, but rather might be inherited or triggered

during early development (Roth et al. 2012). Since these differences were detected in distantly spaced populations with potentially limited gene flow, it is possible that these differences may have evolved as a response to natural selection.

It is unclear, however, whether environment-related differences in memory and the brain may be produced on a smaller geographic scale either through the strength of selection or plasticity. For example, elevation gradients in mountain habitats can produce extremes in winter environmental conditions similar to those along large latitudinal gradients. Along these elevation gradients, winter climate conditions are typically characterized by colder temperatures at higher elevations with much greater and longer lasting snowfall and snow cover. We hypothesized that demands on spatial memory due to higher reliance on food caching in birds at higher elevations should result in enhanced memory supported by an enlarged hippocampus with more neurons and higher neurogenesis rates compared to chickadees residing at lower elevations. To test this hypothesis we compared hippocampal morphology and neurogenesis in wild-caught mountain chickadees from three different elevations, which were sacrificed at the time of capture to avoid potential negative effects of captivity on the hippocampus (LaDage et al. 2009, 2010), and memory performance in laboratory conditions. Mountain chickadees are good candidates for this study because they are permanent residents in our study area in Northern California and do not show altitudinal migration with birds spending the entire winter in coniferous habitats at all elevations (Mccallum et al. 1999, VVP, personal observations).

METHODS

Subjects: For this study, we used 48 juvenile mountain chickadees (*Poecile gambeli*) collected during September 27th - October 10th, 2010 at three different elevations in the Sierra Nevada Mountains in northern California. First, we collected 20 chickadees at high (2400m) and 20 at mid (1800m) elevations, which were separated by 10km. Of these, 8 birds from each elevation were immediately sacrificed for the brain analysis. The remaining 12 birds per elevation were brought into the laboratory for behavioral analyses. In addition, we collected 8 more chickadees from low elevation (1200m, 80 km away) to expand our comparison and immediately sacrificed them for the brain analysis. At each elevation, birds were trapped at four locations using mist-nets in combination with feeders and call playbacks.

We specifically chose to sample first year birds in their first autumn because our explicit predictions are that the differences in memory and hippocampal morphology should be present before any selective event such as winter can result in differential mortality. When we sample juvenile birds before the winter, we generate a random sample of birds that have settled in their winter flocks (Mcculum et al.1989) before any winter-related mortality. Therefore, any differences detected would not reflect a response to winter-like harsh conditions, but might rather be permanent or triggered by some environmental or developmental differences during summer-early fall. In addition, our previous studies with black-capped chickadees detected large differences in hippocampal morphology in birds also collected in the fall during the peak of food caching (Roth & Pravosudov 2009; Roth et al. 2011).

Captive birds from high and mid elevations were housed in 60 x 42 x 60cm wire cages and were visually isolated. Individuals from the two elevations were partitioned in an alternated arrangement of 'high/mid/high/mid' along each row of cages. Each cage design was identical and food and water with vitamins was provided ad libitum. All individuals were fed a diet of seeds (pine nuts and sunflower seeds), wax worms, mealworms, crickets and Orlux Insect Pattee. All individuals were held on a winter photoperiod (9L: 15D) for the duration of the study, which ended on January 10th, 2011.

Testing Room: Caching rates and spatial memory tests were conducted in a testing room adjacent to the bird housing rooms. An opening in the wall connected each individual cage to the testing room. Movement from home cages to the testing room was prompted using light manipulation in the housing and in the experimental room (Pravosudov & Clayton 2002).

The testing room (218 x 373 x 263 cm) contained four trees that each contained 20 evenly spaced cache sites (80 total). Each site consisted of a perch and a drilled caching hole. Each caching hole could be covered with a knot at the end of the sting attached above the hole. An individual would have to remove the knot in order to inspect the hole (Pravosudov & Clayton 2002).

In addition to the caching sites in the trees, 15 caching blocks were placed on each of the two walls (30 total). The opposite walls were arranged identically. Caching blocks (9.0 x 14.5 x 4.0cm) contained a perch and a single drilled hole for caching. Suspended above the cache sites were knotted stings identical to the strings on the trees in order to cover the caches sites. Overall, the room contained 110 caching sites. Prior to all testing, each

individual was allowed to habituate to this testing room for roughly one hour a day on every third day for a total of three hours per individual.

Caching rates: We measured caching rate in all chickadees between November 20th - 27th, 2010, approximately two hours after the lights were turned on in the morning.

Individuals were deprived of food for one hour prior to lights off the previous night and during the experiment. During the test a bowl full of pine nuts and sunflower seeds was provided in the testing room. We recorded the type and amount of food consumed and cached as well as cache locations. All observations were made through a one-way glass window. Each test lasted 20 minutes after which the individual was returned to their home cage. All cached food was removed from sites between individuals. Individuals were tested over three trials occurring every other day and the data were summed over these trials.

One-trial associative learning task: A one-trial associative learning task was conducted between November 29th - December 10th, 2010, each starting two hours after the lights were turned on in the morning. Individuals were deprived of food for one hour before the lights off the previous night and leading up to the experiment. One caching site was chosen at random and openly baited with a high value food item (wax worm) while all other sites remained open. Individuals could find the food but were not allowed to eat it. Once the food was discovered, the individual was allowed to peck at it for a few seconds and then was removed from the testing room. After a 20 min retention interval the bird was allowed back into the testing room with all sites now closed using the knot at the end of a sting. The number of sites inspected until the individual found the food item was

recorded. Individuals were tested once every other day for three trials and memory performance was averaged over these three trials.

Repeated associative learning task: A repeated associative learning task was conducted between December 11th 2010 – January 10th, 2011, starting two hours after the lights were turned on in the morning. Individuals were deprived of food for one hour before the lights off the previous night and leading up to the test. A rewarding cache site (baited with wax worm) was chosen semi-randomly (each individual had a unique site) and kept constant throughout the test. Trials were conducted every other day. During the three pre-trials the individual was allowed into the testing room with all sites open and the reward clearly visible. The individual was allowed to find and eat the food. After the three pre-trials, all sites were covered and the number of inspected sites was recorded for each individual to find the food. All individuals were tested every other day for five trials. After the fourth and fifth trials, when performance of both groups converged, we tested the birds once more on the last, sixth trial after a two-week retention interval to test for potential differences in memory retention and decay.

Brain analyses: Brains were sectioned at 40 μm using a Leica cm3050s cryostat. Every 12th section was used for Nissl staining (Fig. 1A, 1B), while the other sections were stored in cryoprotectant at -80C until further processing (Pravosudov & Clayton 2002, Roth & Pravosudov 2009, Roth et al. 2011, 2012). Hippocampal formation (Hp) volume and hippocampal neuron numbers were estimated using every twelfth Nissl-stained brain section using stereological methods implemented in STEREOINVESTIGATOR software (MBF Biosciences) in conjunction with a Leica microscope (M4000B). Both the

telencephalon volume (Te) and Hp were measured in their entirety, using Te as a control for overall brain size. To estimate Hp and telencephalon volumes we used Cavalieri procedure with the grid size 200 μm (Hp) and 1200 μm (Te) following our previous studies with chickadees (Pravosudov & Clayton 2002, Roth & Pravosudov 2009, Roth et al. 2011, 2012). We used the optical fractionator method to count the number of neurons using a 250 μm grid, 30 x 30 μm counting frame, 5 μm dissector height and 1 μm guards. Following our previous studies (Pravosudov & Clayton 2002, Roth & Pravosudov 2009, Roth et al. 2011, 2012) and widely accepted neuron identification guidelines (e.g. Barnea & Nottebohm 1994, Sherwood et al. 2006), we identified neurons in Nissl-stained tissue as cells exhibiting clear nucleoplasm, one or two darkly stained nucleoli and lightly colored proximate extensions of dendritic processes. The left and right hemispheres were measured independently and summed to produce the report total values.

Neurogenesis: We used doublecortin as a marker of neurogenesis following our previous studies (Fig. 1C, 1D; Chancellor et al. 2011, LaDage et al. 2010, Roth et al. 2012). The doublecortin protein (DCX) is associated with neuronal cell microtubule machinery localized only in new immature neurons (review and references in LaDage et al. 2010). In birds, new neurons appear to express doublecortin for approximately the first 25-30 days of neuronal life and so measuring doublecortin-labeled neurons provides an estimate of a population of all new neurons up to 30 days of age (see LaDage et al. (2010) for detailed discussion of doublecortin staining for measuring neurogenesis). Overall, DCX-staining provides a combined measure of new neuron production and survival (LaDage et al. 2010). We measured doublecortin staining on every twelfth section as in our previous studies (Chancellor et al. 2011, LaDage et al. 2010, Roth et al. 2012).

Briefly, tissue was washed in Tris-buffered saline (TBS) and then incubated in 30 % hydrogen peroxide and TBS (1:50) for 30 minutes at room temperature. Tissue was then washed in TBS and allowed to incubate at a room temperature for 30 minutes in blocking buffer (normal horse serum 1 : 33.3, TX-100 1 : 39.8, and TBS). The sections were then incubated overnight for approximately 18 h at 4C in anti-doublecortin antibody (made in goat; 1 : 200; Santa Cruz Biotechnology, Santa Cruz, CA, USA, SC-8066) and blocking buffer. After incubation, the tissue was washed in TBS and incubated for two hours at room temperature in biotinylated horse anti-goat antibody in blocking buffer (1 : 200, Vector Laboratories, Burlingame, CA, USA, BA-9500). The tissue was again washed in TBS and incubated at room temperature for one hour in an ABC Elite kit (Vector Laboratories, PK-6100), followed by a DAB nickel kit (Vector Laboratories, SK-4100) for two minutes and 18 seconds at room temperature. The tissue was then washed one final time in TBS and then mounted onto slides. These slides were dried overnight at 37.8 C and then Nissl-stained. We also tested for non-specific staining by replacing the anti-doublecortin antibody with TBS during the overnight incubation. Elimination of the primary antibody resulted in no stained cells, which suggests that our main protocol specifically stained cells expressing doublecortin (Fig. 1), rather than staining non-specifically. Boundaries for the Hp were determined per previous studies (Pravosudov & Clayton 2002, Roth & Pravosudov 2009, Roth et al. 2011, 2012). We used the optical fractionator method to estimate the total number of doublecortin-stained cells with the counting frame set to 70 x 70 µm, 250 µm grid and 5 µm dissector height as optimized in our previous studies (Chancellor et al. 2011, LaDage et al. 2010, Roth et al. 2012). The

left and right hemispheres were measured independently and summed to produce the report total values.

Statistical Analysis: We used t-tests and General Linear Models to analyze behavioral results, and General Linear Models and an ordered heterogeneity test (Gaines & Rice 1990) to test whether there was a significant trend between hippocampal structure and elevation following our previous work (Roth & Pravosudov 2009).

Ethical Note: Birds were collected under the state (802024-01 - California) and federal (MB022532) scientific collecting permits. The research reported here adheres to the ASAB/ABS Guidelines for the Use of Animals in Research and all legal and institutional requirements. All animal procedures were approved by UNR IACUC (#00492).

RESULTS

Chickadees from higher elevations had significantly larger hippocampal volume (ANOVA with telencephalon as a covariate, $F_{2,20} = 3.57$; $p = 0.04$; $r_s = 1$; Ordered Heterogeneity Test, $r_s P_c = 0.953$, $p < 0.01$; Fig. 2A) relative to the rest of the telencephalon with significantly higher total number of neurons (ANOVA, $F_{2,20} = 57.9$; $p < 0.001$; $r_s = 1$; Ordered Heterogeneity Test, $r_s P_c = 0.999$, $p < 0.001$; Fig. 1A, 1B, 2B) and more intense hippocampal neurogenesis (the total number of DCX-labeled neurons: ANOVA, $F_{2,21} = 17.4$; $p < 0.001$; $r_s = 1$; Ordered Heterogeneity Test, $r_s P_c = 0.999$, $p < 0.001$; Fig. 1C, 1D, 2C) compared to birds from lower elevations. The number of DCX-labeled neurons relative to the total number of hippocampal neurons did not vary

significantly between birds from different elevations (elevation $F_{2,20} = 0.09$, $p = 0.92$; total number of neurons as a covariate $F_{1,20} = 4.92$, $p = 0.038$), which shows that neurogenesis rates in high elevation birds were higher proportionately to the larger total number of neurons.

Adding telencephalon as a covariate to the analyses of the total number of neurons or the total number of DCX-labeled cells, or removing it from the analysis of hippocampal volume did not change the significant outcomes, which is unsurprising given that there were no significant differences in the telencephalon volume ($F_{2,20} = 0.87$, $p = 0.43$), body mass ($F_{2,21} = 1.45$, $P = 0.256$), or body size (wing length: $F_{2,21} = 1.63$, $P = 0.22$) among the three elevations, suggesting that the elevation-related differences were specific to the hippocampus.

Behavioral tests with captive birds also fully supported these findings: chickadees from the high elevation sites cached significantly more food ($t_{21} = 2.36$, $p = 0.027$; Fig. 3A) and performed significantly better in a one trial associative learning task ($t_{19} = 2.29$, $p = 0.03$; Fig. 3B) when compared to the birds from the mid elevation sites. In the repeated associative learning task with the same reinforced location, chickadees from mid elevations performed significantly worse during the first three trials ($t_{21} = 2.78$, $p = 0.01$; Fig. 3C) converging with the high elevations birds only on the 4th trial. After the two-week retention interval, performance of mid elevation chickadees significantly deteriorated while the performance of the high elevation birds remained at the same level ($t_{21} = 2.44$, $p = 0.02$; Fig. 3C) suggesting faster memory decay in lower elevation chickadees and better memory retention in higher elevation birds. These data show that

chickadees from higher elevations have better spatial memory including memory acquisition and retention compared to birds from lower elevations.

DISCUSSION

Overall, our results showed that higher elevations are associated with enhanced memory and hippocampal structure and that these differences can occur over a small spatial scale. Chickadees from the two closest sampling elevations (high and mid) were only separated by 10 km, yet birds from high elevation had almost double the number of hippocampal neurons and showed significantly better spatial memory performance. These large differences over such a small spatial scale suggest that changes in spatial memory and the brain could track climatic conditions along an elevation gradient in the mountains. Therefore, any changes in climate may potentially trigger changes in cognition and the brain, which may have important implications for the widely reported warming trends along an elevation gradient associated with global climate change (Parmesan 2006). Such a warming trend has the potential to produce significant changes in these birds' cognition and brain structure and in order to fully understand how it may affect population dynamics it will be essential to determine the specific mechanisms of elevation-associated differences in memory and the hippocampus.

Our study showed that even though the total number of new immature neurons varied significantly among birds from different elevations, this variation was proportionate to the variation in the total number of neurons. The number of new immature neurons relative to the total number of neurons did not vary significantly, which suggests that the

higher neurogenesis rates in chickadees from higher elevations may be associated with the maintenance of the larger total number of hippocampal neurons.

Previous studies suggested that hippocampal neurogenesis rates might vary seasonally in food-caching chickadees (Hoshooley et al. 2007). However, multiple studies produced conflicting results with one showing potentially higher neurogenesis rates in October (Barnea & Nottebohm 1994), another showing no seasonal differences in hippocampal neuron production rates (Hoshooley & Sherry 2004) and yet another showing higher neurogenesis rates in January (Hoshooley et al. 2007). It is likely that such seemingly inconsistent results may be due to the fact that these studies measured neuronal survival rates after different time intervals (1-2 weeks in both Hoshooley & Sherry (2004) and in Hoshooley et al. (2007) and 6 weeks in Barnea & Nottebohm (2004)), while not measuring neuronal production rates (e.g. cell proliferation rates). Doublecortin staining produces a combined measure of neuron production and survival, which may not necessarily detect small differences in neuronal survival if neuron production rates are seasonally stable. In our study, we detected elevation-related differences in hippocampal neurogenesis rates at the end of September-beginning of October and thus it remains possible that if we sampled birds during different seasons our results may have been different. Nonetheless, our results clearly indicate significant differences in hippocampal neurogenesis rates as indicated by the combined measure of neuronal production and survival during the peak of food caching at all sampled elevations. It may be interesting to compare neurogenesis rates at different seasons to test if there may be an interaction between elevation and season.

It is important to note that mountain chickadees at higher elevations may initiate breeding one to two weeks later than the birds at lower elevations (VVP, personal observations), which potentially means that our high elevation birds may have been 1-2 weeks younger than the birds sampled at lower elevations. Adult neurogenesis has been reported to decline with age (Barnea & Pravosudov 2011) and so it may be possible that the differences in neurogenesis in our study may have been affected by these rather small potential differences in age between chickadees from different elevations. We, however, do not think it is likely that differences in only 1-2 weeks in age may produce such extremely large differences in neurogenesis rates in birds older than 3 months. Age-related declines in neurogenesis in mammals have been detected using much longer time intervals (usually several months; e.g. McDonald & Wojtowicz 2005) and there appear to be no data on potential differences in neurogenesis over just a few weeks either in mammals or in birds. It was impossible to determine the precise hatch date for all of our birds and so we chose to sample at the peak of food caching, an ecologically relevant time period. Similarly, we think it is even less likely that the differences in hippocampal volume or the total number of hippocampal neurons between chickadees from different elevations were due to these potential small age differences in just 1-2 weeks.

Previous studies of seasonal variation in neurogenesis and hippocampal morphology in chickadees either detected no significant differences in neurogenesis rates, hippocampal volume and the total number of neurons over a 6-month period (Hoshooley & Sherry 2004), or actually reported an increase in 2-week neuronal survival rates from October to January (Hoshooley et al. 2007), showing that even several months of age would not likely result in reduced neurogenesis rates. Our own data on black-capped chickadees

shows no significant differences in the total number of DCX-labeled neurons and in the total number of hippocampal neurons between juvenile birds sampled in mid September (even earlier than in this study) and 8.5-month-old chickadees sampled in February (Roth et al. 2012). Given all this data, it seems unlikely that a difference of just 1-2 weeks in age might have produced our detected differences in hippocampal neurogenesis rates or neuron numbers. Clear differences in spatial memory performance detected in older birds also suggest that potential small age differences between chickadees from different elevations were likely insignificant.

In our previous large-scale comparison of food-caching black-capped chickadees, we determined that environment-related differences in memory and some hippocampal properties such as neuron number and neurogenesis rates (as determined by the total number of DCX-labeled new neurons) were not directly produced by an individual's experiences, but rather may have been inherited or produced during early development (Roth et al. 2012). Because of the large spatial scale of that comparison, the gene flow between these populations may be reduced (Sandoval 1994, Kisel & Barraclough 2010) creating a potential for selection to act on brain properties and select for enhanced memory and a larger hippocampus. Significant differences in mountain chickadees occur on a much smaller spatial scale with a larger potential for gene flow between birds from different elevations. Potential gene flow between different elevations in our system, however, may not prevent strong selection on spatial memory and the hippocampus. Nonetheless, it is equally plausible that the observed differences might be a result of phenotypic plasticity.

Our previous work with black-capped chickadees suggests that some of the variation in the total number of hippocampal neurons might be heritable as chickadees reared and maintained in laboratory conditions had similar numbers of neurons compared to wild birds (Roth et al. 2012). In mountain chickadees, several months of captivity also did not affect the total number of neurons (LaDage et al. 2009). Combined, these data suggest that population differences, at least in the number of hippocampal neurons, are unlikely to result from potential differences in environment-dependent experiences. Nonetheless, present data does not allow for establishing the exact mechanisms behind the elevation-related differences in memory and hippocampal morphology.

Although plasticity remains a viable mechanism underlying the detected differences in hippocampal morphology (Clayton & Krebs 1994, Clayton 2001, Woollett & Maguire 2011), we think that several lines of evidence may potentially argue against this explanation. (A) Previous work on hippocampal plasticity by Clayton and colleagues (Clayton & Krebs 1994, Clayton 2001) showed that only extremely small, memory-based experiences are needed as a switch for the final hippocampal enlargement in developing juvenile birds while any additional memory-based experiences do not result in additional hippocampal enlargement or addition of new neurons. (B) We have previously shown that environmental conditions may affect hippocampal volume, but not the number of hippocampal neurons in mountain chickadees (LaDage et al. 2009). (C) We have previously shown that the differences between black-capped chickadee populations in the total number of hippocampal neurons and neurogenesis (as defined by the number of immature DCX-stained neurons) are unaffected by the post-fledging environment in a common-garden experiment suggesting that the number of hippocampal neurons may

have some genetic basis (Roth et al. 2012). (D) The differences in neuron numbers between birds from different elevations are almost twofold, yet they are present in juvenile birds before their first winter and therefore likely before any large differences in winter climate are experienced.

It is also possible that differences in hippocampal morphology in both black-capped and mountain chickadees could stem from maternal, early developmental or epigenetic effects. Future work should focus on elucidating the exact mechanisms for these differences among the potential suspects: genetics, epigenetics, maternal effects, environmental effects during development and environment-related experiences (plasticity). The nature of these differences may also be important because the rate of response to climatic/environmental changes will depend on the specific mechanism involved. If these changes result from plasticity, birds will be able to adjust to climate changes rather quickly. If, on the other hand, these changes were, at least partially, based on genetic differences, multiple generations would be required to successfully adjust to a changing climate.

Irrespective of the potential causes for the elevation-related differences in memory and the hippocampus, our study shows that environmental differences on a small spatial scale may produce a strong effect on cognition and the brain. In addition, these findings parallel our previous multi-population work showing that differences in winter climatic conditions predict differences in memory and the hippocampus in food-caching birds. Taken together, our data show that this association is robust both along the latitudinal,

longitudinal, and altitudinal gradients in winter climate severity, suggesting that climate may strongly affect memory and the hippocampus in small food-caching birds.

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FIGURE LEGENDS:

Figure 1.

Nissl-stained neurons (A and B) and DCX-stained neurons (C and D) in the hippocampus of the mountain chickadees from high (A and C) and low (B and D) elevations.

Figure 2.

Relative hippocampal volume (A), total number of hippocampal neurons (B) and total number of DCX-labeled neurons (C) in mountain chickadees from three elevations (high = 2400m (n = 8), mid = 1800m (n = 8), low = 1200m (n = 8)). Relative hippocampal

volume is represented by the Least Square Means from the GLM with telencephalon as a covariate. Error bars represent S.E.

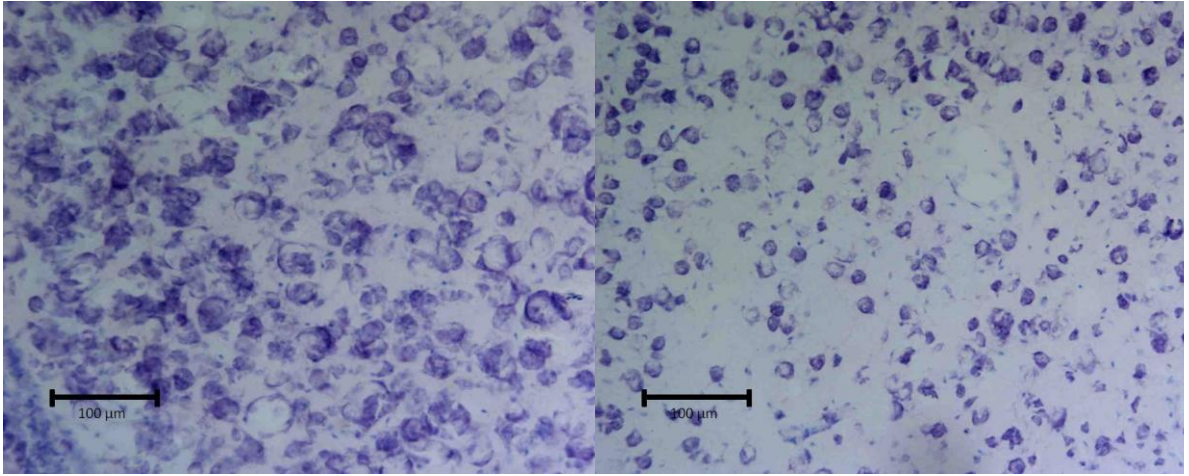
Figure 3.

Caching rates (A), one-trial associative learning task performance (B) and repeated associative learning task performance (C) in two groups of chickadees from high (2400m) and mid (1800m) elevation. Error bars represent S.E.

Figure 1.

A

B



C

D

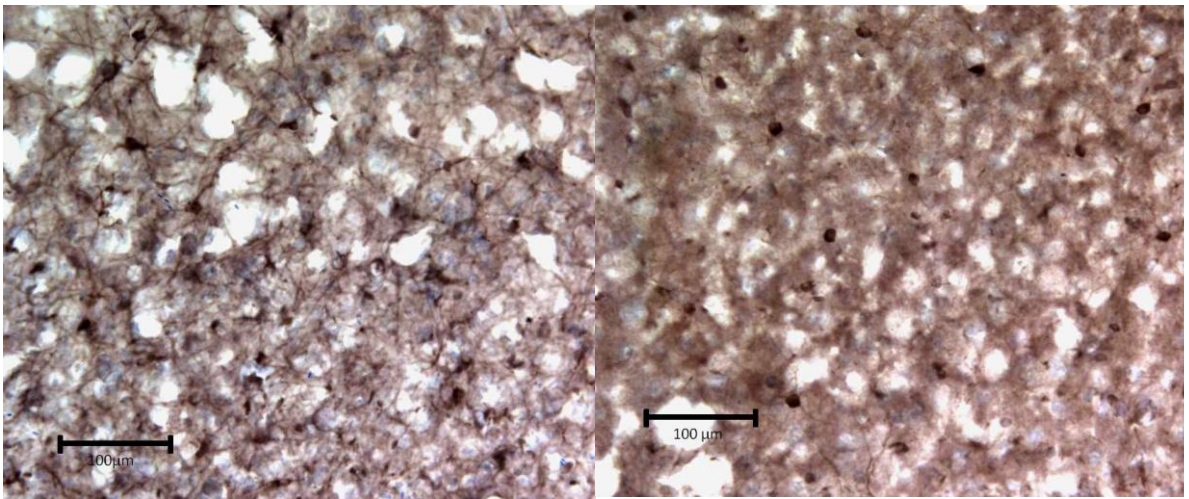
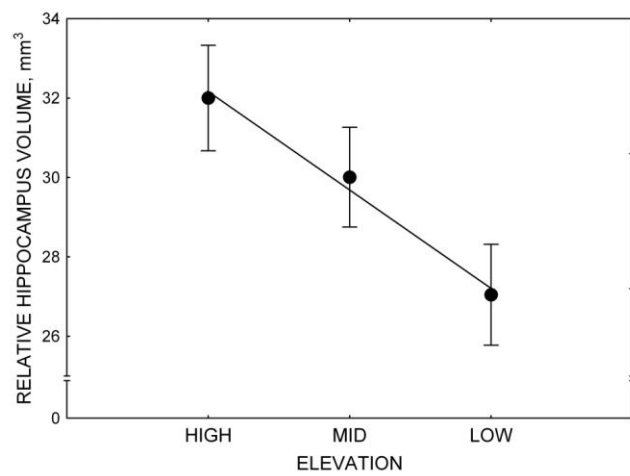
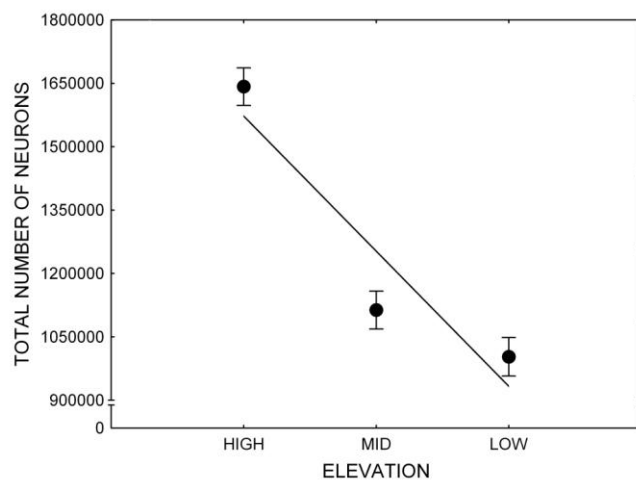


Figure 2.

A



B



C

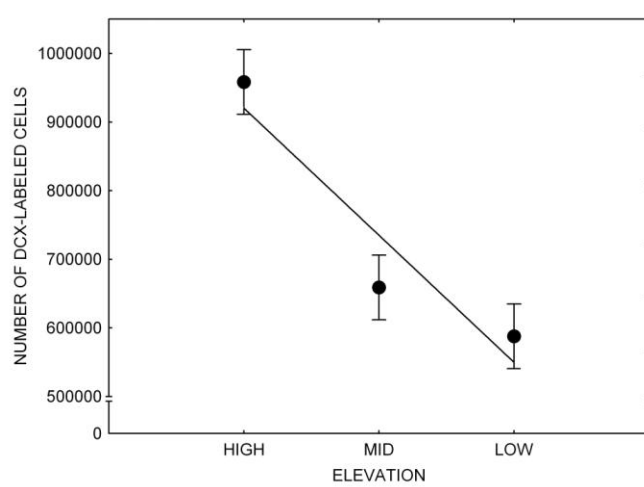
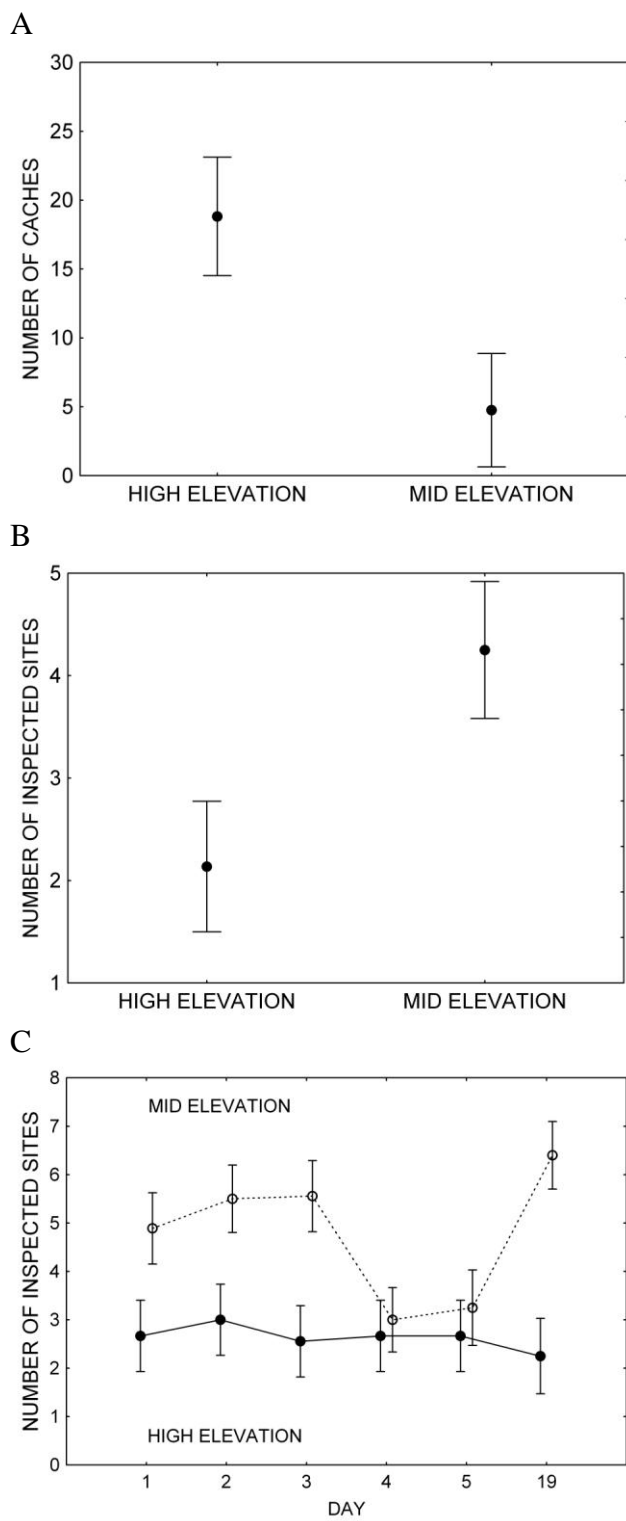


Figure 3.



Chapter 3

Title: Hippocampal neuron soma size is associated with population differences in winter climate severity in food-caching chickadees

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Citation

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ABSTRACT

1. Differential demands on cognitive ability may be expected to result in the evolution of cognition and associated changes in underlying neural mechanisms. While most comparative studies of cognition have focused on volumetric brain measurements it remains unclear whether neuron morphology, which appears to be directly linked to cognitive functions, may be responsive to differential selection on cognitive ability.

2. Food-caching birds rely on caches to survive winter and use spatial memory to recover previously stored food. Birds in more harsh winter climates have been hypothesized to be more dependent on cached food and therefore their winter survival may be expected to be more memory dependent relative to their conspecifics from the milder winter climates. Here we show that neuron soma size in the hippocampus, a brain region involved in memory function, exhibits significant population variation associated with different environmental pressures on spatial memory related to differences in winter climate harshness in two species of food-caching chickadees. Comparing ten populations of

black-capped chickadees and three populations of mountain chickadees along a gradient of winter climate harshness, we found that birds from harsher environments had significantly larger hippocampal neuron soma sizes.

3. Using chickadees from the two most divergent populations reared in a laboratory environment, we showed that these differences appear to be at least partly heritable as significant differences between these populations remained in birds sharing the same laboratory environment. At the same time, laboratory reared birds had significantly smaller neuron soma size compared to the wild-sampled birds, suggesting that at least some variation in neuron soma size may be due to environment-related plasticity.

4. Our data suggests that environment-related selection on memory may generate differences in neuron morphology, which appear to be controlled by some heritable mechanisms and likely underlie population differences in spatial memory.

Key Words: memory, hippocampus, neuron size, evolution, climate, chickadee, food caching

INTRODUCTION

Cognition and variation in cognitive traits are likely to play an important role in evolutionary processes (Dukas 2004). Environmental differences that generate differential selection pressures on cognitive traits might be expected to result in the evolution of cognitive differences along with differences in the underlying neural mechanisms of these traits (Sherry 2006). At the same time, enhanced cognition can allow animals to successfully invade new environments, as well as to survive perturbations associated with changing environments (Sol et al. 2005). As cognition is a

product of neuronal processes (Dukas 2004), selection on cognitive traits is expected to produce some changes in the neural structures supporting those cognitive traits. Much of the research on the evolution of cognition and especially comparative studies of cognition, relies heavily on volumetric/size measurements of the brain or some specific regions of the brain in order to understand the evolution of enhanced cognition (Healy et al. 2007; Roth et al. 2010). Yet it is frequently unknown which specific neurobiological mechanisms can produce these volumetric changes (Healy et al. 2007; Roth et al. 2010). Recent studies argue that volumetric variation of the brain does not scale well with variation in cognitive abilities and that the total number of neurons appears to be a much more accurate predictor of cognitive ability (Herculano-Houzel 2011, 2012). Indeed, cognitive functions such as learning and memory appear to be based on the synaptic activity of specific neurons (Chen et al. 2012; Reijmers et al. 2007; Kandel & Schwartz 1982), yet evolutionary comparative studies rarely investigate whether and how specific selection pressures might be associated with differences in neuron morphology.

Food-caching species have been used extensively to investigate how environmental pressures related to food caching are associated with evolutionary changes in spatial learning abilities and the hippocampus, a brain region known to be involved in spatial learning (Krebs et al. 1989; Sherry et al. 1989). While initial studies compared the relative hippocampal volume of food-caching and non-caching species (Krebs et al. 1989; Sherry et al. 1989), more recent approaches involved within-species population comparisons (Pravosudov & Clayton 2002; Roth & Pravosudov 2009; Roth et al. 2011, 2012; Freas et al. 2012).

Many food-caching species exist across large gradients of environmental conditions, which were hypothesized to be associated with differential dependence on food caches and thus on spatial memory used for cache retrieval (Pravosudov & Clayton 2002). Most food-caching species are non-migratory and winter in temperate environments, which exhibit large differences in winter climate severity on both small and large geographical scales. Populations wintering in more severe environments with colder and longer periods of winter conditions are hypothesized to be more highly dependent on cached food than populations wintering in more moderate climates, especially in small bodied species such as chickadees (Pravosudov & Clayton 2002; Roth & Pravosudov 2009). Winter periods are characterized by lower ambient temperatures, which require more energy intake to meet potentially increased metabolic demands, yet naturally available food is unpredictable and usually in short supply. Longer winters and lower ambient temperatures increase metabolic requirements, which should result in a need for more caches and more successful cache retrieval for survival. Cache retrieval is spatial memory dependent and therefore harsher environments likely produce stronger selection pressures on spatial memory, which is hippocampus-dependent. Therefore, it can be predicted that environments with more severe winter climate should favor the evolution of enhanced spatial memory, which could be achieved via neurobiological changes in the hippocampus.

Our previous studies supported this prediction by showing that more severe winter conditions are associated with enhanced spatial memory, an enlarged hippocampus, higher total number of hippocampal neurons, and higher hippocampal neurogenesis rates on a large continental scale in black-capped chickadees, *Poecile atricapillus* (Pravosudov

& Clayton 2002; Roth & Pravosudov 2009; Roth et al. 2011, 2012; Chancellor et al. 2011), and on a small spatial scale along an elevation gradient of winter climate severity in mountain chickadees, *P. gambelli*, in the Sierra Nevada (Freas et al. 2012).

Furthermore, these population differences in black-capped chickadees appear to be dependent on some heritable mechanisms, at least in some populations as shown in a ‘common garden’ experiment (Roth et al. 2012).

The total number of neurons in the brain appears to be a more accurate predictor of cognitive abilities than the volume of the brain (Herculano-Houzel 2011, 2012) suggesting that enhanced cognition in populations experiencing increased selection pressure on cognitive traits could be achieved via the larger number of neurons.

However, it is equally important to understand whether evolutionary processes affecting cognition might also generate differences in neuron morphology (for example via affecting its developmental program), yet little is known about population/species variation in neuron morphology associated with specific selection pressures.

Neurons are essential for learning, and dendritic and synaptic structures of neurons are known to be directly involved in learning and memory function (e.g. Kandel & Schwartz 1982, Kolb & Whishaw 1998). Therefore, it is plausible to hypothesize that selection on neuronal functions, such as learning and memory could indirectly affect neuron properties involved in these functions, assuming these properties have some heritable mechanism(s).

In this study, we focused on neuron soma size, which is suggested to be associated with cognitive ability (e.g. De Voogd & Nottebohm 1981; Oberlander et al. 2004). In songbirds, seasonal changes in volume of song control brain nuclei involved in song

learning are associated with changes in neuron soma area (e.g. Tramontin et al. 2000, Thompson & Brenowitz 2005). Montagnese et al. (1993) reported that two food-caching bird species had larger calbindin-immunoreactive cells in the hippocampus compared to two non-caching species. Schizophrenia and major depressive disorder, which, among many other things, are also known to be related to reduced cognitive ability, have been associated with reduced neuron soma size in the hippocampus and in the anterior cingulate cortex in humans (Benes et al. 1991; Jonsson et al. 1999; Cotter et al. 2001; Stockmeier et al. 2004). In zebra finches (*Taenopygia guttata*), neural aromatization resulted in enhanced spatial memory acquisition and in larger neuron soma size in the hippocampus (Oberlander et al. 2004). Larger neuron soma could contain larger cellular and metabolic machinery needed to serve a larger neuron dendritic tree, more synaptic connections, and higher neuron activity (e.g., De Voogd & Nottebohm 1981, Kolb & Whishaw 1998, Oberlander et al. 2004). Larger memory ability is associated with more dendritic arborization and a larger dendritic tree (e.g., Kolb & Whishaw 1998), therefore larger soma size may be associated with memory via supporting such increased dendritic arborization and neuron activity. Higher neuron activity is dependent on more mitochondria, which appear to originate in neuron soma (Kann & Kovacs 2007). Therefore, it might be hypothesized that neuron soma size could be reflective of memory and learning ability with larger neuron soma supporting enhanced memory and learning ability.

The goal of this study was to investigate whether environmental differences associated with differential demands for memory-dependent food caches are associated with differences in neuron morphology in hippocampal neurons assessed by estimating neuron

soma area. We used brain tissue from the birds collected for previously published studies and compared 10 populations of food-caching black-capped chickadees and 3 populations of mountain chickadees previously shown to be significantly different in spatial memory, hippocampal volume, total number of hippocampal neurons, and hippocampal neurogenesis rates (Roth & Pravosudov 2009; Roth et al. 2011, 2012; Freas et al. 2012; Chancellor et al. 2011). Additionally, to investigate whether any potential population differences in hippocampal neuron soma area might be due to plasticity related to local environmental differences and/or whether these differences might be controlled by some heritable mechanisms, we analyzed previously collected brain tissue and compared the two most diverse populations of black-capped chickadees that were reared and maintained in the same laboratory environment ('common garden') and compared them to those from the wild (Roth et al. 2012). We specifically predicted that birds from more harsh environments should have a larger hippocampal neuron soma size that potentially support their enhanced spatial memory ability and that an increased soma size in these populations is a result of increased selection on spatial memory and/or a plastic response to environmental differences.

METHODS

Subjects

Neuron soma area measurements were newly conducted on brain tissue collected for our previous studies of black-capped and mountain chickadees (Roth & Pravosudov 2009; Roth et al. 2011, 2012; Freas et al. 2012).

Black-capped chickadees: wild sampled populations

We used brain tissue from 120 black-capped chickadees that were collected from 10 populations on a large continental scale along both longitudinal and latitudinal gradients of winter climate severity for our previous studies of hippocampal morphology (Roth & Pravosudov 2009; Roth et al. 2011). These locations have been ranked on winter climate severity based on average air temperature over winter (Roth & Pravosudov 2009; Roth et al. 2011): Alaska-Fairbanks (AKF) < Alaska-Anchorage (AKA) < Maine (ME) < British Columbia (BC) < Minnesota (MN) < Montana (MT) < Iowa (IA) < Colorado (CO) < Kansas (KS) < Washington (WA). These locations can also be roughly separated into two groups, one having harsh winter environments (AKF, AKA, ME, BC, MN) and the other exhibiting mild winter environments (IA, CO, KS, IA, WA) with MT falling in the middle as we have done in our previous analyses of population genetics, winter climate, and hippocampal morphology (Pravosudov et al. 2012).

At each location, we trapped 12 birds using mistnets during late September-early October and perfused all birds in the field to avoid any potential captivity effects on the brain (Roth & Pravosudov 2009; Roth et al. 2011). Brains were extracted immediately following perfusion and shipped to the laboratory at the University of Nevada, Reno where they were processed, frozen, and then sectioned at 40 μm . Every 4th section was mounted on slides and Nissl-stained (for all detailed procedures see Roth & Pravosudov 2009; Roth et al. 2011).

Mountain chickadees: wild sampled populations

We used brain tissue from twenty-four mountain chickadees that were collected at three elevations (8 birds per elevation, 2400 m, 1800 m and 1200 m) along an elevation gradient of winter climate severity in the Sierra Nevada in northern California for our previous study on the relationship between winter climate and hippocampus morphology (Freas et al. 2012). At each elevation, wild chickadees were captured with mistnets during late September-early October and sacrificed on the same day to avoid any captivity effects on the brain. Birds were perfused and their brains were extracted, processed, frozen, and sectioned at 40 μm . Every 4th section was mounted on slides and Nissl-stained (Freas et al. 2012).

Black-capped chickadees: common garden experiment

To investigate whether any potential differences in hippocampal neuron soma area were due to plastic responses to immediate environmental differences and/or whether these differences were due to some potentially heritable mechanisms, we used brain tissue from 24 black-capped chickadees that were previously collected from the two extremely different populations (around Anchorage, Alaska – 12 birds and around Manhattan, Kansas – 12 birds) and reared and maintained under the same laboratory ‘common garden’ environment (Roth et al. 2012). All chickadees were collected at approximately 10 days of age before their eyes were open while the chicks were in a dark nest cavity (Roth et al. 2012). From the time of collection, chicks from both populations were hand-

reared and maintained in the same common environment for their entire life until they were sacrificed for the brain analyses at approximately 8.5 months of age. Each of the 24 birds used in this study came from a different nest and all details about hand-rearing and maintenance have been published previously (Roth et al. 2012).

All birds were perfused and their brains extracted, processed, frozen and sectioned at 40 μm following the exact procedures used to process brains from wild-sampled chickadees (Roth & Pravosudov 2009; Roth et al. 2011). Every 4th section was mounted on slides and Nissl-stained (Roth et al. 2011, 2012).

In addition to comparing brain tissue from these ‘common garden’ birds from two populations, we also compared them to the brain tissue from chickadees that were previously wild-caught in the same populations (Roth et al. 2012).

Estimating hippocampal neuron soma area

Neurons were identified in Nissl-stained tissue as cells that exhibited one or two darkly stained nucleoli, a clear nucleoplasm, and lightly colored extensions of dendritic processes, (Fig. 1) using the same procedure as in our previous studies (Pravosudov & Clayton 2002; Roth & Pravosudov 2009; Roth et al. 2011, 2012), and widely accepted neuron identification guidelines (e.g. Barnea & Nottebohm 1994; Sherwood et al. 2006).

Neuron soma area measurements were taken from every 9th mounted section (every 36th section) starting with the second section used previously to measure hippocampal volume (Roth & Pravosudov 2009; Roth et al. 2011, 2012; Freas et al. 2012). Overall we

measured neuron soma area on a minimum of 4 and a maximum of 6 hippocampal sections. On each section, we divided hippocampal tissue into three approximately equally sized areas and sampled one randomly chosen location within each area on both left and right hemispheres (Fig. 1). Such sampling ensured random coverage of the entire hippocampal area, but we did not focus on any specific regions of the hippocampus. Once the location was chosen, we used a frame (75 x 110 μm) to insure unbiased sampling of all neurons and measured neurons independent of their size by selecting and measuring the first five neurons within the frame by moving left to right, beginning at the left side of the frame. The criterion for selecting the first neuron was that it should either cross the left line of the frame or be located to the right of the left line. If there were fewer than five neurons within the frame, we moved the frame to the right for as long as it took to identify the five required neurons.

For each selected neuron, we traced the cell area using StereoInvestigator software (MBF Biosciences, Williston, VT, U.S.A.), which estimated neuron soma area based on the tracing. It is important to note that cells are likely not positioned in the same way within the sections, but, assuming random orientation of cells within the hippocampus, measuring the soma area across multiple randomly selected cells should accurately reflect the average area of the neuron soma. Our sampling scheme resulted in measurements of 15 neurons for each hemisphere for each measured tissue section for a total of 30 neurons for each tissue section. Overall, we estimated neuron soma area for a minimum of 120 and a maximum of 180 hippocampal neurons per bird. All of these measurements were averaged to produce one mean neuron soma estimate per bird, which was used in the statistical analyses. While there may be several neuron types in the avian hippocampus,

our study did not differentiate between them. We measured all cells as long as they met our criteria for neuronal phenotype and our sampling covered the entire hippocampal formation; our measurements reflect an average neuron soma area across all neuron types and all hippocampal regions. All measurements were done blind to the identity of each bird by CAF.

We used previously published estimates of telencephalon volume in the same individuals (Roth & Pravosudov 2009; Roth et al. 2011, 2012; Freas et al. 2012) to test for relative differences in hippocampal neuron soma area.

We also measured neuron soma in two other telencephalon regions – the mesopallium (M), across the ventricular zone from the hippocampus and the hyperpallium apicale (HA), adjacent to the hippocampus (Fig. 1) in the two populations (Washington and Alaska-Anchorage) that showed the largest differences in hippocampal neuron soma area. We applied the same methods by sampling three randomly chosen locations and sampling 5 neurons. Overall, we estimated HA neuron soma area for a minimum of 90 and a maximum of 150 neurons per bird and M neuron soma area for a minimum 120 and a maximum of 180 neurons per bird.

There were no significant effects of sex in any of the comparisons and therefore sexes were pooled.

Statistical analysis

We used General Linear Model (GLM) to test for differences among populations and treatment groups. For all comparisons, we tested for potential differences by adding

telencephalon volume (to control for potential scaling of neuron soma area with the brain size) as a covariate, as well as by using raw neuron area with no covariates. Variation in hippocampal volume is generally associated with variation in telencephalon volume (e.g. Pravosudov & Clayton 2002) and using telencephalon as a covariate would allow disassociating any existing population effects on hippocampal neuron soma area from any potentially confounding effects associated with overall brain size. To test for the predicted relationship between winter climate severity and hippocampal neuron soma area, we used an ordered heterogeneity test (Gaines & Rice 1990) that specifically tests for ordered associations as we have done in our previous studies (Roth & Pravosudov 2009; Freas et al. 2012).

RESULTS

Black-capped chickadees: wild populations along longitudinal and latitudinal gradients of winter climate severity

There was significant variation in hippocampal neuron soma area relative to the telencephalon size among ten black-capped chickadee populations over a large continental scale ($F_{9,109} = 11.53$, $P < 0.001$; Telencephalon as a covariate: $F_{1,109} = 21.3$, $P < 0.001$). There was also a significant relationship between winter climate severity and relative neuron soma area (Fig. 2A,B, 3A, $r_s = 0.915$, Ordered Heterogeneity Test, $r_s P_c = 0.915$, $k = 10$, $P < 0.001$), with birds from populations with more severe winter climates having larger mean neuron soma size relative to telencephalon volume. This relationship

remained highly significant ($F_{9,110} = 13.68$, $P < 0.001$; $r_s P_c = 0.96$, $P < 0.001$) when telencephalon volume was not used as a covariate.

When we separated all ten populations into two groups containing five independent populations and sharing either a relatively harsh (Alaska-Anchorage, Alaska-Fairbanks, British Columbia, Maine, and Minnesota) or a relatively mild winter environment (Kansas, Washington state, Colorado, Iowa and Montana), there were also significant differences in the hippocampal neuron soma area (t -test, $t_8 = 3.89$, $P = 0.005$).

Finally, we tested whether population differences in hippocampal neuron soma size were independent of any potential differences in neuron soma size in the rest of the telencephalon by comparing the two most divergent populations (AKA and WA) and using mean neuron soma size in HA and M as covariates. Population differences in hippocampal neuron soma size remained highly significant with HA neuron soma size as a covariate ($F_{1,21} = 41.5$, $P < 0.001$, HA neuron soma area – $F_{1,21} = 15.09$, $P < 0.001$) and with M neuron soma size as a covariate ($F_{1,21} = 43.42$, $P < 0.001$, M neuron soma area – $F_{1,21} = 4.17$, $P = 0.054$). At the same time, both HA ($F_{1,21} = 8.19$, $P < 0.01$) and M ($F_{1,21} = 7.53$, $P = 0.012$) neuron soma areas were also significantly larger in Alaska birds compared to birds sampled from milder climates in Washington state.

Mountain chickadees: wild populations along an elevation gradient of winter climate severity in the mountains

The significant relationship between more severe winter climate and larger neuron soma area was also present in mountain chickadees along the elevational gradient; larger hippocampal neuron soma area relative to telencephalon size was associated with higher elevations (Fig. 2C,D, 3B, General Linear Model, $F_{2,20} = 5.06$, $P = 0.02$, Telencephalon as a covariate, $F_{1,20} = 5.1$, $P = 0.03$; Ordered Heterogeneity Test, $r_s = 1$, $P_c = 0.98$, $k = 3$, $P < 0.01$). Differences among the three elevations remained significant when telencephalon was not used as a covariate ($F_{2,21} = 3.83$, $P = 0.038$).

Black-capped chickadees: common garden experiment

We compared the neuron soma area of chickadees reared and maintained in a controlled laboratory environment and chickadees from the same populations (Kansas and Alaska Anchorage) that were trapped directly from the wild (Roth et al. 2011, 2012). There were significant differences between the two populations ($F_{1,43} = 30.89$, $P < 0.001$), as well as between chickadees sampled directly from the wild and birds reared and maintained in the same laboratory conditions ($F_{1,43} = 6.21$, $P = 0.016$). The interaction between population (Kansas vs. Alaska) and condition (wild vs. lab reared) was not significant ($F_{1,43} = 2.06$, $P = 0.16$), but telencephalon size was positively related to neuron soma area ($F_{1,43} = 7.18$, $P = 0.01$). Chickadees from Alaska had relatively larger hippocampal neuron soma area than birds from Kansas independently of their condition (wild vs. common-garden), but lab reared and maintained chickadees also had smaller relative hippocampal neuron soma area than their conspecifics sampled directly from the wild

(Fig. 4). These results remained significant ($P < 0.05$) even if we did not use telencephalon as a covariate.

DISCUSSION

Our study showed that there is a significant association between winter climate severity and hippocampal neuron soma area in both black-capped chickadees on a large continental scale and in mountain chickadees on a small spatial scale along a montane gradient. Since variation in hippocampal neuron soma size mirrors predicted population differences in reliance on spatial memory, our findings suggest that larger neuron soma may subserve enhanced spatial memory. Secondly, our study showed that population differences in hippocampal neuron soma size appears to be controlled at least partially by some heritable mechanisms, because differences between black-capped chickadees from the two most divergent populations remained significant even when birds were reared and maintained in the same laboratory conditions. In addition to having some heritable basis, neuron soma size also appears plastic in response to environmental conditions, most likely as a result of potential GxE interactions, as lab-reared birds had smaller neuron soma size than birds sampled directly from the wild.

Compared to chickadees from Washington state, neuron soma areas in HA and M were significantly larger in Alaska birds, these locations representing the two most divergent populations showing the largest differences in hippocampal neuron soma size, suggesting that other brain regions might also be associated with differences in winter environmental conditions. However, the relationship between neuron soma size and winter climate appears to be significantly stronger specifically in the hippocampus, as the differences in

the hippocampal neuron soma size between these populations were highly significant relative to the mean neuron soma area in at least two other telencephalon regions (HA and M). Alaska chickadees' hippocampal raw neuron soma area was 51% larger than that of Washington chickadees while such difference was only 23% for HA neuron soma size and 12% for M neuron soma size.

Hippocampal neuron soma area was significantly and positively associated with the telencephalon volume suggesting that individuals with larger brains have larger hippocampal neurons. While this association is noteworthy, it was not critical for our main conclusions as the relationship between winter climate and hippocampal neuron soma area was highly significant even when raw neuron soma area was used in the analyses.

Our results provide support for the hypothesis that a harsh winter environment is associated with increased selection pressure on spatial memory needed for successful cache retrieval. It appears that such pressure likely resulted in the observed significant differences in spatial memory, hippocampal volume, total number of hippocampal neurons, and adult hippocampal neurogenesis rates among different populations of two species of food-caching chickadees inhabiting different environments. This study shows that these differences extend to the hippocampal neuron morphology, with birds in harsher environments tending to have significantly larger neurons relative to the neuron soma size in the rest of the telencephalon. This is a significant finding that indirectly suggests that enhanced spatial memory in chickadees from populations under increased selection on memory-based cache retrieval might be, at least partially, subserved by

neurons with larger soma size that likely provide the increased cellular metabolic machinery needed to subserve a larger neuron dendritic tree and/or more neuron activity (e.g. Kolb & Whishaw 1998).

It is interesting that larger hippocampal neuron soma size in birds from environments with a more severe winter climate is also associated with significantly higher total number of hippocampal neurons as well as with significantly higher adult hippocampal neurogenesis rates (Roth & Pravosudov 2009; Roth et al. 2011, 2012; Chancellor et al. 2011; Freas et al. 2012). These data suggest that it is unlikely that larger neurons in birds from harsher environments may simply be a reflection of their relatively older age, as birds with larger neurons also appear to have higher neuron turnover rates. While we could not differentiate potentially different neuron types in the hippocampus, we specifically measured all cells that exhibited neuronal properties irrespective of their size, so our estimates should be an accurate representation of an average neuron soma size in the entire hippocampal formation.

The observed large population variation in hippocampal neuron soma size (or at least part of it) could be potentially explained by environmentally induced plasticity. All wild-caught birds in this study were sampled during early fall when environmental conditions were relatively mild for all of the compared populations and therefore, it is unlikely that population differences in neuron soma size were simply due to immediate differences in severity of winter conditions when memory may be especially important. Nonetheless, it is possible that at least some of the population differences in neuron soma size may be due to differences in food caching intensity, which is usually highest during the autumn

months (Pravosudov 2006) and which likely involves heavy spatial memory used to encode memory for numerous cache locations (Male & Smulders 2007). This possibility is also reinforced by the data showing that birds reared and maintained in a laboratory environment had significantly smaller neuron soma area compared to the wild-sampled conspecifics from the same populations.

However, our comparison of the ‘common garden’ birds that showed significant population differences in neuron soma size despite sharing the same laboratory environment from 10 days of age suggests that neuron soma size also appears to be controlled by some heritable mechanisms involved in neuron development, which may have a genetic basis. Combined with such potential heritable mechanisms, differences between wild-sampled and lab-reared birds, as well as population differences in hippocampal neuron soma size suggests that hippocampal neuron soma size is both plastic to some degree and also determined by some heritable, possibly genetic, mechanisms so that the overall variation in soma size is likely a product of some GxE interactions.

It remains plausible that not all of the reported population differences can be explained by heritability, as we performed our ‘common garden’ experiment with only the two most divergent populations and only in black-capped chickadees. As a result, it is possible that differences in neuron soma size between populations of both mountain and black-capped chickadees over small spatial scales were entirely due to environment-induced plasticity. We also could not rule out potential maternal and/or epigenetic effects because our ‘common garden’ birds were collected at 10 days of age. Nonetheless, differences

between populations shown in a ‘common garden’ experiment lend more support to the hypothesis that population differences in hippocampal neuron soma size could be a result of differential selection pressures on memory associated with environment-dependent reliance on memory-based food caching (Roth et al. 2012; Pravosudov et al. 2012).

Assuming that natural selection can act on memory ability, such selection can indirectly produce significant changes in the neural structures underlying memory as long as these structures are controlled by some heritable mechanisms.

It is interesting that birds reared and maintained in the same laboratory environment had significantly smaller hippocampal volume compared to the wild-sampled birds from the same populations, yet there were no significant differences in the total number of hippocampal neurons (Roth et al. 2012). The present study, on the other hand, showed significant reduction in hippocampal neuron soma size in the lab reared birds, which suggests that changes in hippocampal volume were at least partially due to changes in neuron soma size and not the total number of neurons.

Overall, our results showed that increased winter climate severity is associated with an enlarged neuron soma area in the hippocampus in two species of food-caching chickadees and that population differences in neuron soma size appear to be based on some heritable mechanisms. While our data do not explain the exact mechanism of how increased neuron soma area may be related to spatial memory, it points at neuron soma as a potential mechanism involved in memory function. Our findings that population differences in neuron soma size are associated with differences in spatial memory indirectly suggest that enhanced spatial memory in birds from harsher winter

environments may be subserved by hippocampal neurons with larger soma size. Since larger soma size may provide increased cellular and metabolic machinery needed to maintain larger dendritic arborization and higher neuron activity, chickadees from harsher environments may potentially be able to store more memories associated with the larger number of caches. The data reported here on hippocampal neuron soma size add significantly to the mounting evidence that environmental conditions in food-caching birds are associated with differential selection pressure on memory-dependent cache retrieval, resulting in significant modifications of the neural mechanisms, including hippocampal neuron soma size, which appears to support enhanced spatial memory. While environment and experience-induced plasticity appear to explain at least some of the observed population variation in neuron soma size, population genetics (Pravosudov et al. 2012) and ‘common garden’ experiments (Roth et al. 2012, this study) suggest that such variation is consistent with a history of natural selection. Nonetheless, future studies are needed to establish whether the observed population differences in neuron soma size have a genetic basis or whether they are caused by either maternal or epigenetic effects.

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FIGURE LEGENDS

Figure 1

Sampling schematics. The hippocampal formation was roughly subdivided into three sampling areas. Additional sampling control areas were within mesopallium (M) across the ventricle from the hippocampus and hyperpallium apicale (HA).

Figure 2

Hippocampal neurons (photographed in the middle of the hippocampus in roughly the same area for all comparisons) in (A) black-capped chickadees from one of the most severe winter climates (Alaska) and (B) from the mildest winter climate (Washington state) and in mountain chickadees from high elevation (C) and low elevations (D) in Sierra Nevada mountains in northern California.

Figure 3

The relationship between hippocampal neuron soma area and winter climate severity in ten populations of black-capped chickadees (A) and in mountain chickadees from three

elevations (B). Soma area values are least square means from General Linear Model with telencephalon volume as a covariate.

Figure 4

Mean hippocampal neuron soma area in wild sampled (filled circles) and in lab reared ('common garden'; open circles) black-capped chickadees from Kansas and Alaska-Anchorage.

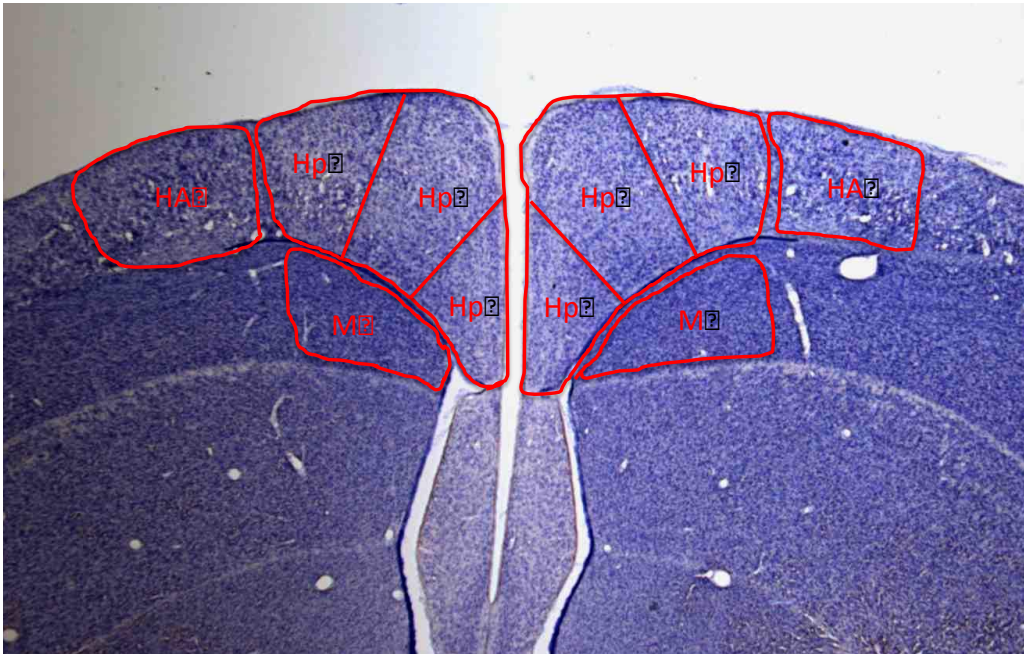
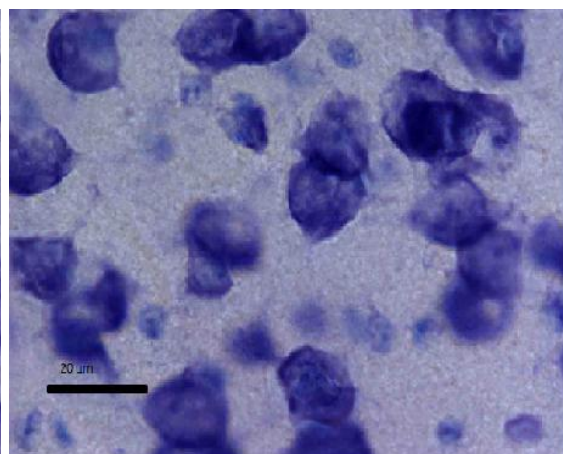
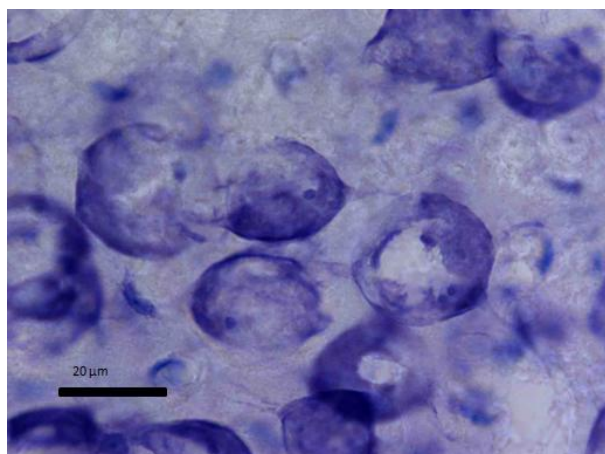
Figure 1.

Figure 2

A. BCCH: Alaska-Anchorage

B: BCCH: Washington State



C. MOCH: High Elevation

D: MOCH: Low Elevation

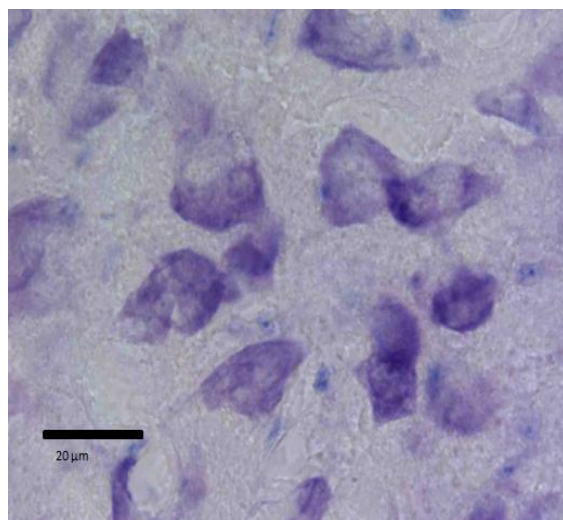
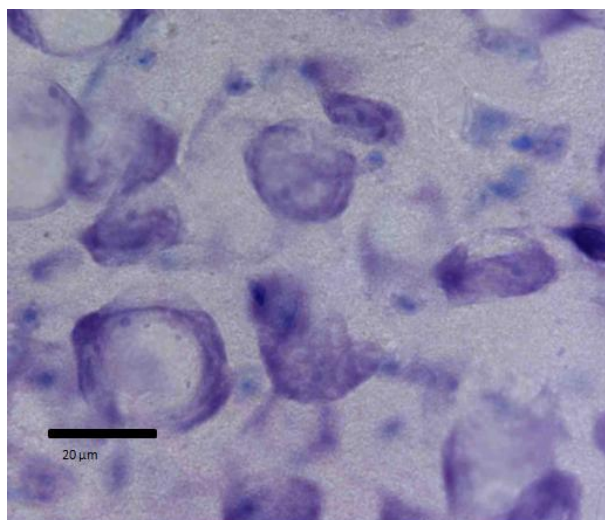


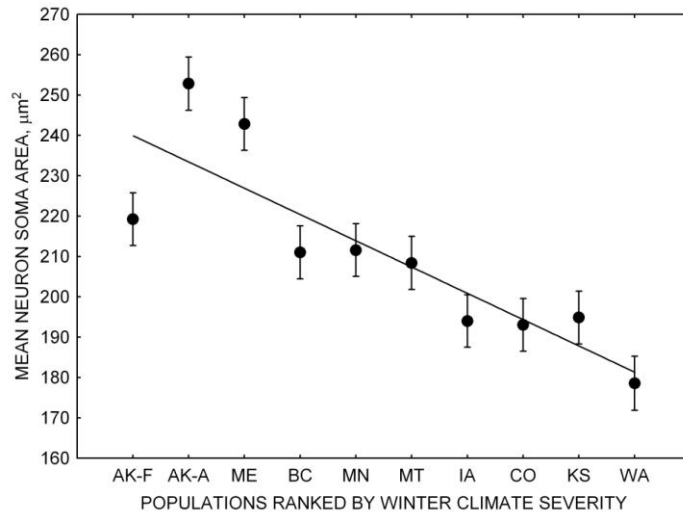
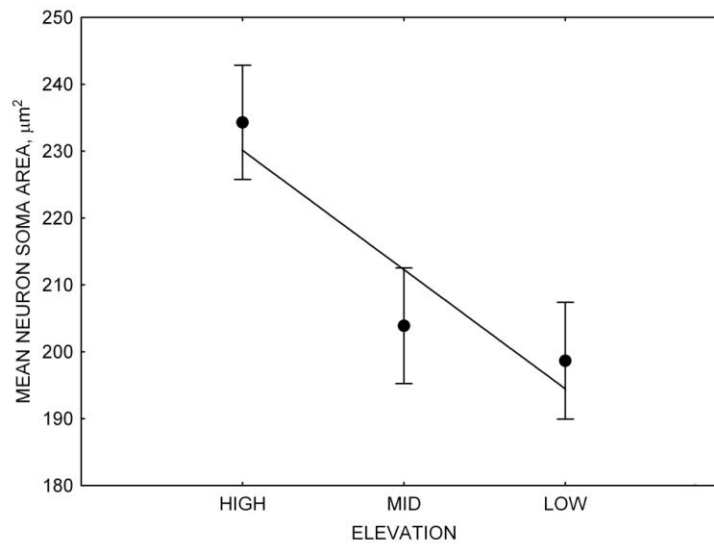
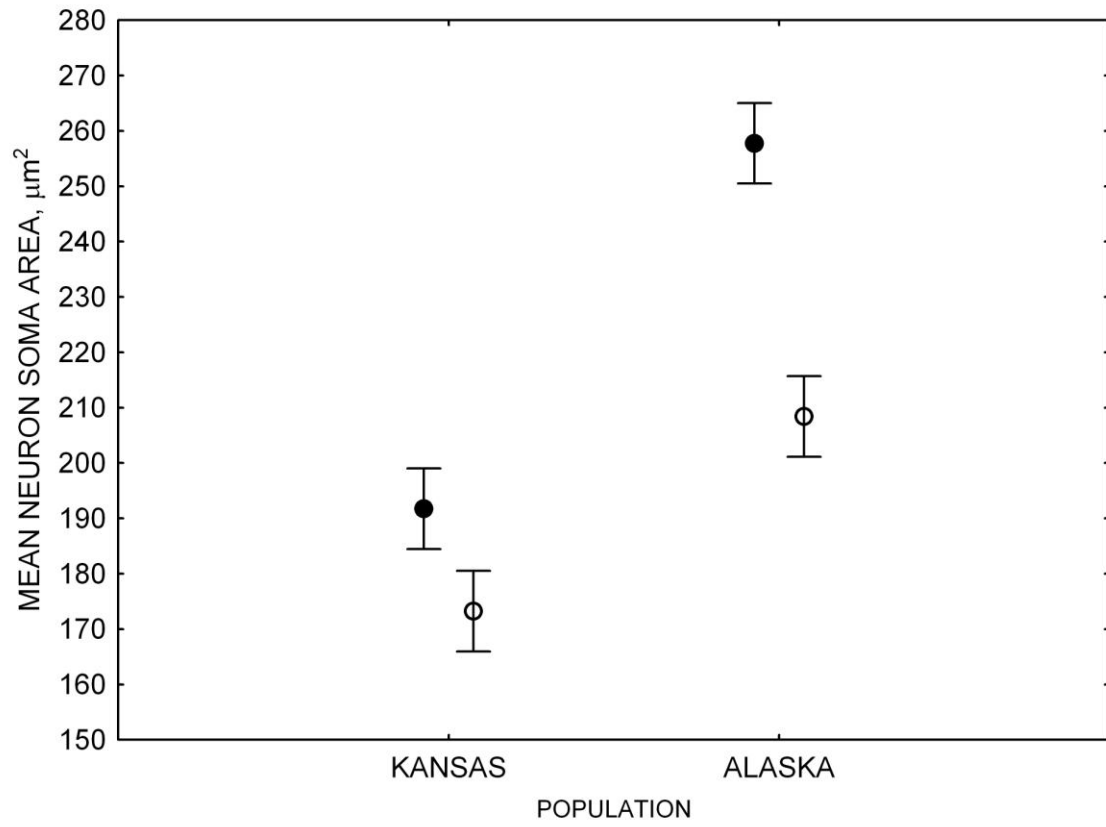
Figure 3.**A. Black-capped chickadees****B. Mountain chickadees**

Figure 4.

Chapter 4

Conclusions

In our small spatial scale study in Mountain Chickadees, we predicted that individuals from higher elevations experience more harsh winter climates and would in turn exhibit enhanced spatial memory and enhanced neural structure in the hippocampus. We found that High elevation individuals cached significantly more, and performed better on both the one-trial associative learning test and the repeated associative learning test when compared to individuals from Mid elevation. There was a strong relationship between enhanced spatial memory in the behavioral testing and the underlying neural architecture in the hippocampus. There is a robust positive relationship between elevation and the architecture of the hippocampus. Individuals from higher elevations had larger hippocampi with more hippocampal neurons, and higher levels of hippocampal neurogenesis compared to birds from the Mid and Low elevations. These results support all of our predictions and suggest that the same observed relationship between environmental harshness and spatial memory in food-caching birds on a large geographic scale can also be observed across a much smaller geographic scale with similarly large differences in winter climate. The findings suggest that winter climate harshness may cause large selection pressures on food-caching animals causing populations living in these environments to exhibit large behavioral and morphological differences compared to populations living in more mild climates.

In this work we also hoped to look at the possibility that neuron morphological characteristics are involved in variation observed between populations and the plastic

response of the hippocampus when individuals live in captivity. We predicted that hippocampal neuron soma size would be positively related with other neural enhancements that track with environmental harshness from our previous studies. We found a robust positive relationship between neuron soma size and populations of two species of chickadees ranked by environmental harshness along multiple spatial scales. By analyzing the two populations of lab-raised black-capped chickadees representing the most divergent climates we observed a significant reduction in hippocampal neuron soma size in both populations suggesting that soma size is plastic. However, there was still a significant difference between populations suggesting a heritable component. Our results suggest that larger soma sizes may be an additional morphological trait that could potentially be measured in spatial memory studies. Hippocampal neuron soma sizes may in fact be a better indicator of spatial memory enhancement compared to hippocampal volume as differences between divergent populations are maintained under laboratory conditions even with the observed plasticity of the trait.

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