THE EFFECTS OF HOUSING CONDITIONS ON NEUROGENESIS IN THE BLACK-
CAPPED CHICKADEE

by

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ABSTRACT

Black-capped chickadees (*Poecile atricapillus*) store and retrieve thousands of seeds during the fall and winter, seemingly in anticipation of reduced resource availability coinciding with onset of harsh weather, and laboratory experiments have confirmed the necessity of hippocampal integrity in successful performance of this specialized ability. These birds undergo changes in rates of hippocampal neurogenesis throughout the year; however, results from laboratory experiments designed to gain further insight to this phenomenon have been equivocal. In this study, we were interested in teasing apart the effects of two factors associated with housing conditions of captive birds: housing size and housing location. To do this, we used aviary and cage housing in indoor and outdoor settings, across season. We found that birds housed indoors experienced a decrease in hippocampal neurogenesis compared to those housed outside; however, there was no difference between birds housed in cages compared to those housed in aviaries. Results from this study suggests that a transition from nature to an indoor environment has greater consequences on hippocampal neurogenesis than does a reduction in space. Results from this study will help to inform researchers of ideal laboratory conditions for further experimentation with this model.

LIST OF ABBREVIATIONS USED

MWM	Morris Water Maze
BrdU	Bromodeoxyuridine
DCX	Doublecortin
NeuN	Neuronal specific nuclear protein
MAM	Methylazoxymethanol acetate
HA	Hyperpallium apicale
Hp1	Medial hippocampus
Hp2	Dorsomedial hippocampus
CWS	Canadian Wildlife Service
OA	Outdoor aviary
IA	Indoor aviary
OC	Outdoor cage
IC	Indoor cage
UCCA	University Committee on Laboratory Animals
CCAC	Canadian Council on Animal Care
PBS	Phosphate buffered saline
PBS/T	Triton-X and PBS solution
ir	Immunoreactivity

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Introduction

The potential influence of environment on brain and behaviour highlights the importance of experimental control in psychological experiments, particularly when studying animals captured from the wild. Specifically, it is critical to understand the influence of the potentially drastic change in environment when an animal is transported from its natural habitat to laboratory housing, independently and in combination with other experimental manipulations. Thus, studies of the effects of captivity on brain and behaviour are needed. My study examines the effects of captivity on brain structure and neurogenesis in a songbird model, the black-capped chickadee (*Poecile atricapillus)*. By teasing apart the influence of variables encountered by wild black-capped chickadees subjected to research environments, results from this study will inform researchers of how their experiments may be influenced by the transition from nature to laboratory.

Environmental Enrichment

In contrast to field experiments, laboratory experiments provide necessary control over extraneous factors to manipulate and examine variables of interest. However, in doing so, a subject's housing environment may be austere compared to that of free-living counterparts. To counter this, experimenters often provide environmental enrichment to animals in home cages. Environmental enrichment is typically defined as laboratory housing sporting any permutation of the following factors: increased space, increased complexity, temporal variability, and group versus individual housing. The addition of items such as toys, tunnels, and running wheels is common in animal husbandry and is thought to eliminate stress and abnormal repetitive behaviour in laboratory housed

animals. The ways in which these environmental manipulations affect an animal's brain and behaviour remains a popular topic in psychology and neuroscience.

Seminal research by Hebb (1949) and Rosenzweig (1966) formed the modern framework for research aimed at understanding the influence of physical and social environments on brain and behaviour. In their classic studies, Hebb and Rosenzweig found that subjecting rats to complex and novel environments lead to measurable changes in their brains and behaviour compared to rats housed in temporally stable laboratory cages. Rosenzweig (1966) housed post-weaning aged rats for 80 days in either enriched or impoverished environments and subsequently measured their brain weight, cortical thickness, and enzymatic activity. He found that rats housed in enriched environments had a 4% increase in cortex weight, a 6% increase in cortical thickness (neglecting the outer layer), a slight increase in acetylcholine (AChE) activity, and an 8% increase in cholinesterase (ChE) activity. Though simple in design, these early experiments were groundbreaking in that they offered insight to the relationship between early life environment and brain morphology and function--a finding that would later be expanded to include plasticity during adulthood.

Effects on Behaviour

In an investigation of the behavioural effects of environmental enrichment, Kempermann, Kuhn, and Gage (1997) tested spatial memory abilities of rats subjected to enriched (i.e. equipped with a nest, tunnels, toys, and running wheel) and standard (i.e. smaller cage with no enriching items) laboratory housing during the first 40 days after birth. To test spatial learning, the authors employed a Morris Water Maze (MWM) task requiring animals to swim to a submerged platform; the length of swim path from the

entry point of the maze to the platform and the amount of time taken to reach the platform were measured. Animals from enriched environments outperformed those from standard environments on both measures of spatial learning ability through the first four days of training, though the difference between groups on these measures was negligible by the fifth and final day of testing.

Nilsson, Perfilieva, Johansson, Orwar, and Eriksson (1999) extended the association between environmental enrichment and learning by demonstrating that positive effects of enrichment can occur even when the enrichment is introduced during adulthood. In their study, the authors assigned nine-weeks post-weaning aged rats to either enriched or impoverished environments for four to eight weeks, similar to the condition described above. Over four days following the housing period, rats were subjected to the same MWM task as previously mentioned, and the authors found that subjects from enriched environments outperformed their impoverished counterparts. Further evidence for the relationship between environmental enrichment and learning and memory has been unequivocal (Pham, Söderström, Winbald, & Mohammed, 1999; Frick & Fernandez, 2003; Bruel-Jungerman, Laroche, & Rampon, 2005; Bell, Livesey, & Meyer, 2009; Birch, McGarry, & Kelly, 2013), and the positive effects of enrichment on learning and memory extend to different types of learning, such as operant discrimination (Bourgeon, Xerri, & Coq, 2004).

Contributing Factors

Because environmental enrichment typically includes a number of elements, it can be difficult to attribute its effects to a single enriching variable. For instance, most enriched environments, as compared to standard housing conditions, present opportunity

for increased motor activity; thus, it is difficult to attribute observed effects of enrichment to factors such as social interaction or novelty, when increased motor activity or exercise alone can account for observed changes.

Having established relationships between complex environments and brain and behaviour, Ferchmin, Bennett and Rosenzweig (1975) sought to examine individual contributions of each enriching variable. In these experiments, they found that rats (approximately 25 days post-weaning) permitted to observe, but not participate in, complex environments and rats exposed to social interaction as their sole element of enrichment did not experience the previously reported effects of environmental enrichment. On the other hand, physical exercise alone is sufficient to elicit positive effects of environmental enrichment. For example, Fordyce and Wehner (1993) subjected otherwise sedentary adult mice (three months old) to one hour of treadmill running, five days per week, for eight weeks. The authors reported a 2- to 12-fold performance enhancement on spatial learning on both a MWM and a place learning-set task. The idea that an interaction between enriching elements is required to elicit the maximum benefit from enrichment persists in more recent studies (e.g. Harburger, Nzerem & Frick, 2007).

Taken together, the studies reviewed here established a concrete link between environmental enrichment and behaviour and brain morphology, and suggest that exercise is a key factor in eliciting positive effects of enrichment. With this knowledge, research shifted toward investigation of the specific neurobiological changes leading to measurable changes in brain structure and function. The specific neurobiological underpinnings of environmental enrichment will be reviewed next.

Neural Substrates

Given the complexity of neurons, with their dendritic branching and polysynaptic axonal connections, the idea of their replication seems unlikely; thus, the earliest theories regarding the mechanistic effects of environmental enrichment precluded structural changes (Gage, 2002). Hebb (1949) first forwarded the idea that strengthening of neural networks underlies learning. In his view, strengthening of synapses occurs between neurons of pre-existing networks, rather than through structural changes in these networks via integration of new components. Though this hypothesis gained significant support from electrophysiological data (Bliss & Gardner-Medwin, 1973), with increasing evidence for the relationship between environmental enrichment and brain morphology (e.g. increased brain weight), there remained some findings for which this theory could not account. The explanation for those findings would have to come from the idea that there are indeed incorporations of addition components: new neurons.

Neurogenesis

Neurogenesis involves the proliferation, differentiation, migration, recruitment, and integration of new neurons in the central nervous system. Neural stem cells proliferate in specific regions of the brain, and through a host of genetic, molecular and environmental factors, these cells develop into mature neurons sporting structural and functional characteristics necessary for successful integration to neural circuits. The primary region of proliferation is the subventricular zone (SVZ). Cells then migrate from this region of birth to the regions where needed through recruitment. Finally, the mature neurons are integrated into existing neural circuits (see Zhao, Deng & Gage, 2005 for review). Not all neurons live, thus it is important to consider not only rate of cell birth in

the SVZ, but rate of survival, by quantifying how many neurons born actually make it to integration.

Quantification of Neurogenesis

The earliest method to appear in literature as a marker of neurogenesis was thymidine-H[3]administration (Smart & Leblond, 1961). This marker is a radioactive nucleoside that becomes incorporated into DNA during the S-phase of the cell cycle and can later be visualized through autoradiographic imaging. However, due to its radioactive properties, this method has received much criticism, suggesting that it may lead to cell cycle arrest and apoptosis (Yanokura, Takase, Yamamoto & Teraoka, 2000). A similar method involves administration of bromodeoxyuridine (BrdU), a synthetic nucleoside and thymidine analog, which also becomes incorporated to the DNA of dividing cells during replication. This method remains popular among researchers investigating neurogenesis; however, although it is not radioactive, BrdU can also cause cell death through its mutagenic properties (Taupin, 2007).

A number of proteins have been implicated in various stages of neurogenesis. Doublecortin (DCX) is a microtubule-associated protein that is expressed in immature, migratory neurons and post-migratory neurons undergoing plastic changes (Brown et al., 2003; Nacher, Crespo, & McEwen., 2001). Unlike thymidine-H[3] and BrdU, since DCX is neuron specific, it does not require co-labeling with a neuronal specific protein such as neuron specific nuclear protein (NeuN). As an endogenous maker, DCX also eliminates the need for injections, which in themselves can be stressful and may interfere with cell proliferation.

Foundations of Research in Neurogenesis

Since mature neurons are too complex to replicate through cell division, a theory suggesting that environmental enrichment and associated learning processes rely on addition of new neurons to neural networks entails that new neurons must be derived from neural stem cells. Altman (1962) sought to investigate whether brain lesions caused generation of new brain cells in rats, and became the first to show that new neurons can be found in the brains of adult animals. Using thymidine-H^3, Altman was able to determine which cells were born after its injection into the animal. In addition to the appearance of new glia, Altman found radioactive labeled neurons in the brains of animals sacrificed one month after lesioning. This revolutionary discovery offered some insight to what structural changes are influenced by the effects of environmental enrichment.

Having discovered that new neurons can appear in adult animals, Altman and Das (1964) next set out to determine whether environmental enrichment increases neuron production in the rat brain. While initial data did not reveal an increase in new neurons in the rat cortex as a consequence of environmental enrichment, they did reveal an increase in glia. Subsequent studies suggested that this increase in glia could be attributed to the presence of oligodendrocytes and astrocytes (Imamoto, Paterson, & Leblond, 1977; Walsh, Budtz-Olsen, Penny, & Cummins, 1969).

Using the same labeling method as in their previous study, but without subjecting subjects to an environmental enrichment paradigm, Altman and Das (1965) identified an area in the dentate gyrus of the hippocampus that showed a peak in neuron population around 15 days of life, and a subsequent decline thereafter. The potential for adult

neurogenesis in the dentate gyrus of the hippocampus in rats was further studied by Kuhn, Dickinson-Anson and Gage (1996). In their study, the authors injected six 27 month old rats with BrdU and looked at the fate of these cells either 12h or 4-6 weeks after labeling. Even in 27 month old rats, BrdU positive (+) cells were present in the dentate gyrus 4-6 weeks after injection, though at lower levels than in 6 month old rats. Further, the number of BrdU+ cells in the subgranular zone--where neural progenitor cells are thought to proliferate before migration--was lower in 27 month old rats relative to 6 month old rats 12 hours after injection, suggesting that the age-related decline in neurogenesis is due to decreased proliferation of neural precursors, rather than to decreased differentiation or survival of these cells. Despite a lack of data suggesting an increase in the number of new neurons in brains of enriched animals in these studies, subsequent studies with a narrowed focus on particular brain areas suggest that enrichment does indeed lead to increased neuronal number.

Neurogenesis and Enrichment

As previously mentioned, Kempermann, Kuhn & Gage (1997) found that mice subjected to environmental enrichment had increased spatial memory capabilities; in addition, their study revealed important changes in brain structure. In their study, mice were given daily injections of BrdU during the final 12 days of housing. A sample of mice from each housing group was sacrificed at either 1 or 40 days after their final injections and their brains were collected for histology. The authors found that there was no difference between housing groups on number of BrdU+ cells in the dentate gyrus in the groups sacrificed one day after their final injections, suggesting that enrichment did not affect proliferation of progenitor cells in this region. However, the authors found a

57% increase in the number of BrdU+ cells in mice from enriched environments sacrificed 40 days after their last injections, suggesting that environmental enrichment promoted survival of proliferating neuronal precursor cells in the dentate gyrus. This finding is important in that it demonstrates not only a relationship between environmental enrichment and improved learning ability, but also suggests that this enhancement is supported by an increase in survival of new neurons rather than an increase in proliferation of neurons that are available to be incorporated. However, because all animals showing an increase in neuronal survival underwent spatial memory training, this increase could not be attributed to the environmental manipulation alone. This raised the question of whether learning can elicit neurogenesis.

Revisiting the idea that particular factors differentially affect the brain and behaviour, researchers have been interested in testing individual effects of environmental enrichment on proliferation of neural progenitor cells and survival of differentiated neurons. In their study, van Praag, Kempermann and Gage (1999) subjected three month old mice to one of five conditions: A learning condition in which mice were trained on a MWM task, a swimming condition in which mice were required to swim in the MWM apparatus for the same amount of time as mice in the learning condition, a running group in which mice were permitted ad libitum use of a running wheel, an enriched condition in which 14 mice (versus 3-4 in all other conditions) were housed together in a large cage equipped with a number of tunnels and toys and a single running wheel, and a control condition in which the mice were housed in standard cages with no enriching elements. Mice were injected with BrdU for the first 12 days of the environmental manipulation and sacrificed on either day 13 or 29. Results indicated that on day 13 mice in the running

condition had a greater number of BrdU+ progenitor cells in the subgranular zone of the hippocampus compared to all other groups, with no significant differences between any of the other groups. Further, on day 29, mice in the running and enriched groups showed a 201% and 175%, respectively, increase in BrdU+ cells in the dentate gyrus compared to controls, with no significant differences between the other groups. Results from this study indicate that voluntary physical activity and environmental enrichment are each adequate in approximately doubling the survival of neural progenitor cells, while only voluntary exercise is adequate for increasing the number of progenitor cells in the subgranular zone. Interestingly, participation in a spatial learning task was not sufficient for cell proliferation or survival.

Contrary to this, Gould, Beylin, Tanapat, Reeves & Shors (1999) found that classical conditioning and MWM tasks specifically engaging the hippocampus enhance survival of differentiated neurons. To determine whether training on a spatial or associative learning task enhanced cell survival, the authors injected rats with BrdU either one week before training, or during training and after reaching learning criterion, and sacrificed them either 24h or after learning criterion was achieved. Results showed that rats trained on an eyeblink response task using a trace protocol or those trained on a MWM task using a place-test protocol (both of which are hippocampus-dependent) had more BrdU+ cells in the dentate gyrus than those trained on either an eyeblink response task using a delay protocol or a MWM task using a cue training protocol. These findings suggest that learning can promote survival of adult born neurons, so long as the task engages the hippocampus.

Given that physical exercise can promote proliferation of neural progenitor cells, and that environmental enrichment and hippocampus-dependent learning tasks can promote survival of differentiated neurons, Fabel et al. (2009) sought to determine whether sequential combination of the aforementioned factors can have additive effects on adult hippocampal neurogenesis. To test this idea, the authors housed 8-week old mice for 10 days in a cage equipped with a running wheel, after which time they were switched to an enriched environment (as previously described in Kempermann & Gage, 1999) for 35 days. Control conditions consisted of the following: mice housed in standard cages for the entire duration of the experiment; mice housed in the running wheel condition before being switched to standard housing after 10 days; and mice housed in standard housing without a running wheel before being switched to the enriched environment after 10 days. All mice were injected with BrdU during the last two days of the first phase. At the end of the experiment, the authors harvested subjects' brains and treated tissue with a NeuN antibody to determine which of the BrdU+ cells were neurons. Results showed that all experimental conditions, regardless of when animals had access to the running wheel or experienced enrichment, lead to a greater number of BrdU+ and BrdU+ and NeuN+ co-labeled cells than the control group. The effects were most pronounced in the running/enrichment group, followed by the running/standard and standard/enrichment groups, respectively. Results from this study further elucidate the relationship between particular factors of environmental enrichment and their influences on different stages of neurogenesis, and demonstrate how combination of enriching factors and timing of their presentation can maximize the enhancement of neurogenesis. Particularly, these results demonstrate that physical exercise plays a sizeable role in neurogenesis, and that

combining this form of enrichment with others may have a synergistic effect on neurogenesis, involving the proliferation and survival of new cells, respectively.

Avian Models

Studies reviewed thus far outline important developments in our current understanding of environmental enrichment and its effects on behaviour and brain structure and function using rodent models. These include other sites of adult neurogenesis that have been identified in mammals, including the subventricular zone and the olfactory bulbs. Concurrent to these studies, research on a number of non-mammalian species lends support to homologies between species, thereby strengthening the degree of generalizability of findings. While mammalian models are mainstay in research focussing on environmental enrichment due to ease of genetic manipulation, and homologues to human systems and cellular processes, there are some shortcomings to their use. Particularly, much research using the rat--a nocturnal animal--is carried out during the day, and thus these experiments may lack ecological validity in all applications to humans. Therefore, in addition to the value afforded by mammalian models (e.g. genetic manipulation, implications for humans), it is necessary to look to other models to gain insight of the basic processes of neurogenesis from a comparative evolutionary perspective. By learning more about the relationships between proximate factors of the environment and neurogenesis, we can make predictions about its adaptive value, which will in turn lend insight to the similarities and differences of these relationships across class. One model that has been implemented in this approach is the songbird.

Studies using avian models have proven fruitful in advancing our understanding of the relationships between environment, behaviour, and brain. Different species engage

in specialized behaviours, such as homing in pigeons (*Columba livia domestica*), migration in many species, or food caching in corvids such as crows *(Corvus brachyrhynchos)*, that require learning and memory of large scale space. In addition, songbirds (Oscines, a suborder of the Passeriformes), such as the white-throated sparrow (*Zonotrichia albicollis*) or the dark-eyed junco (*Junco hyemalis*) must learn to produce songs from tutors by listening to and memorizing songs of their fathers, and then practicing these over development to establish an adult, species-typical song, much in the way human infants learn language from parents (Doupe and Kuhl, 1999). These abilities are critical to each particular species' survival and reproductive success and can be influenced by environmental pressures. Naturally, these specialized abilities require a suite of neurobiological underpinnings to host their performance. Here I will focus on two of these: the hippocampus and spatial memory, and the avian song system and song learning.

Homology of Mammalian and Avian Hippocampus

A number of homologies exist between the avian and mammalian hippocampus (Lee, Miyasato & Clayton, 1998). While structural appearance is dissimilar between class, conspicuous differences have been resolved on the basis of immunocytochemical evidence. Indeed, Erichsen, Bingman & Krebs (1991) suggest that the neuron dense, V-shaped layer and surrounding dorsomedial zone of the avian hippocampus correspond to the hippocampus proper and dentate gyrus of its mammalian counterpart, respectively. Further, the avian and mammalian hippocampus contain similar cell types including pyramidal and granule cells (Molla, Rodriques, Calvet & Garcia-Verdugo, 1986; Montagnese, Krebs & Meyer, 1996). While connections with other brain regions are also

similar between class, some differences exist; for example, the avian hippocampus does not have strong projections to the septal region, lacks a postcommisural fornix, and does not receive direct input from the medial septal nucleus (Macphail, 2000, as cited in Broadbent & Columbo, 2000). Despite these differences, most evidence suggests that the two structures are functionally homologous, particularly in their role in spatial memory and learning.

Hippocampus, Learning and Environment

Behaviours requiring spatial memory in wild birds are hippocampus-dependent. Bingman, Ioalé, Casini & Bagnoli (1990) tested hippocampal involvement in homing pigeons' (*Columba livia domestica*) development of navigational maps. They found that young pigeons with lesioned hippocampi, as compared to sham-lesioned birds, were impaired in their ability to gain their homeward bearing when released from unfamiliar locations, suggesting that the hippocampus is critical in the regulation of navigational behaviour in a natural setting.

In this thesis, my model species of choice is the black-capped chickadee (*Poecile* atricapillus). Chickadees are a North American songbird that are widely studied for both their vocal repertoire (Ficken et al 1978), which contains learned vocalizations, and their food storing. Caching and retrieval of food sources is another hippocampus-dependent behaviour. Caching species such as the chickadee store thousands of food items and are able to retrieve them with above chance levels of accuracy, meaning they don't simply store a multitude of items at random in hopes of finding one when needed, but rather remember exact locations of food caches for accurate retrieval later. Sherry & Vaccarino (1989) subjected black-capped chickadees (*Poecile atricapillus*) to bilateral hippocampal

aspiration and assessed their caching and retrieval behaviour. Results showed that hippocampal aspiration reduced cache retrieval to chance levels; however, number of attempts to recover caches was not affected, meaning the memory for cache sites, rather than caching behaviour itself, was hippocampal-dependent. A further experiment assessed birds' abilities on tasks requiring memory for either place or cue. Results from these experiments showed that hippocampal aspiration interrupted the place-memory task, but not the cue-memory task. Together, these experiments demonstrate hippocampal involvement in working memory and memory for places, and that hippocampal integrity is required for cache recovery.

Another interesting aspect of black-capped chickadees is that caching behaviour (among other behaviours) is seasonally variable; that is, the frequency at which chickadees cache and retrieve food depends on environmental pressures that vary with season (see Hoshooley & Sherry, 2010 for review). Typically, birds from North America begin storing seeds at a high frequency during fall months, when food availability is reduced. Studies have shown that along with these seasonal changes in behaviour arise seasonal changes in brain structure and function. Particularly, chickadees experience seasonal changes in hippocampal volume and neurogenesis that correlate with seasonal changes in food-storing behaviour. Seasonal variation in caching behaviour, a specialized hippocampus for food storing and retrieval, a learned vocal repertoire with associated neural regions, and the presence of adult neurogenesis make the black-capped chickadee an ideal model species for this study.

Manuscript Introduction

Black-capped chickadees (*Poecile atricapillus*) store and retrieve thousands of seeds during the fall and winter, seemingly in anticipation of reduced resource availability coinciding with onset of harsh weather (Odum, 1942; Krebs et al, 1989; Sherry, Jacobs, & Gaulin, 1992). Laboratory experiments have confirmed the necessity of hippocampal integrity in successful performance of this specialized ability; indeed, lesions to hippocampi of black-capped chickadees reduces memory of caching locations to chance levels in a lab setting (Sherry & Vaccarino, 1989). However, other factors associated with captivity, such as spatial confinement, reduced activity levels, and social isolation, may also affect hippocampal integrity (Tarr, Rabinowitz, Imtiaz & DeVoogd, 2009).

Adult neurogenesis is a crucial component of the neural substrates of caching behaviour. In their seminal work, Barnea and Nottebohm (1994) found that black-capped chickadees captured in the fall months experienced an increase in neuronal recruitment to their hippocampi compared to birds captured at other times throughout the year. They hypothesized that the addition of new neurons to the hippocampus is part of a process of neuronal replacement required for acquisition of new spatial memories. Complementary to these findings, neuronal number appears stable across season, meaning that any new neuron detected must have 'replaced' an older cell (Hoshooley & Sherry, 2004).

Additional evidence for a relationship between caching behaviour and neurogenesis comes from studies using experimental manipulation of caching. LaDage, Roth, Fox and Pravosudov (2009) demonstrated that restricting caching behaviour by not providing environmental means to do so lead to decreased neurogenesis in hippocampi of

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black mountain chickadees (*Poecile gambeli*). More recently, Hall, Delaney and Sherry (2014) found that pharmaceutical suppression of neuronal recruitment via injection of the toxin methylazoxymethanol (MAM) prior to a spatial learning task led to decreased retrieval accuracy as compared to control birds. Taken together, these studies suggest a causal and experience-dependent relationship between caching behaviour and neurogenesis.

Another neural measure, volume of hippocampus, does not seem to change seasonally. One study by Smulders, Sasson and DeVoogd (1995) found that hippocampi of black-capped chickadees captured during fall months--when these birds generally begin storing food--were larger than those of birds captured at other times of year. However, Hoshooley and Sherry (2007) found that hippocampi of black-capped chickadees captured between February and April were larger than those of birds captured between October and November. Further, Hoshooley et al. (2007) found no differences in hippocampal volume of black-capped chickadees captured throughout the year, despite a relative increase in caching behaviour during October. However, there may be a more specific relationship between caching behaviour and hippocampal volume, rather than seasonal change (Roth and Pravosudov, 2009). They found that hippocampal volume of black-capped chickadees from various geographic regions increased as a function of environmental harshness and caching behaviour necessitated by such conditions. Together, these studies demonstrate inconsistencies in the relationships between environment, caching behaviour, and hippocampal volume and suggest that factors other than climatic harshness and caching behaviour may mediate these relationships.

The effects of spatial restriction and climatic variation on hippocampal neurogenesis and volume require further investigation. In a seminal study investigating the effects of captivity on hippocampal volume and neurogenesis, Tarr, Rabinowitz, Ali Imtiaz and DeVoogd (2009) found that hippocampal volume, but not neurogenesis, was decreased after 4-6 weeks of captivity compared to free flying birds. Upon capture, birds in their study were injected with bromodeoxyuridnie (BrdU)--a synthetic adenosine used as a marker of neurogenesis--and subsequently released into the wild or housed in indoor cages after a 24h acclimation period in outdoor cages. No difference in new cell survival (BrdU+ cells in hippocampus after 4-6 week period) between groups was detected; however, hippocampal volume was reduced by 23% in captive birds. The lack of a difference in neurogenesis in captive birds contradicts findings of Barnea and Nottebohm (1994), in which captive birds showed a 50% reduction in new cell survival compared to free flying birds and no difference was detected in hippocampal volume. There are two ways in which the design of these experiments may explain the apparent discrepancies. First, in their study, Tarr, Rabinowitz, Ali Imtiaz and DeVoogd (2009) captured birds during late November and early December, during which any seasonal peak in neurogenesis would be expected to have tapered off; therefore, the null results here could be due to a floor effect of neurogenesis level, meaning that the base rate of neurogenesis in these birds may have been low enough such that the effect of captivity was not pronounced. In contrast, Barnea and Nottebohm (1994) found that the suppression of neurogenesis in their study occurred during the peak in neurogenesis found in October; therefore, if birds in their study captured in October were removed from analysis, the

effect of captivity on neurogenesis may likewise have been below the threshold for detection.

In terms of hippocampal volume, it may be important to note that the transition from nature to captivity between studies was equivocal in that while birds in the study by Tarr, Rabinoqitz, Ali Imtiaz and DeVoogd (2009) were housed in indoor cages, the birds in the study by Barnea and Nottebohm (1994) were housed outdoors. Given this, the former study represents not only a decrease in space, but also a removal from the natural environment. The transition from nature to indoor housing conditions may have caused more stress than the transition from free-flight to cage, which may explain the reduction of hippocampal volume in the former group.

In the current study, our objective was to tease apart the effects of two factors associated with housing conditions of captive birds. To do this, we used aviary and cage housing in indoor and outdoor settings, across season. In doing so, we were able to consider more precisely the effects of spatial confinement, climate, and their interactions on hippocampal neurogenesis and volume. Further, we will revisit seasonal variability of hippocampal neurogenesis and volume. Results from this study will be important in interpreting results from laboratory studies of learning and memory and other hippocampus-dependent measures in captive chickadees. Results from this study also lend insight to the problem of housing conditions on brain morphology and function and behaviour in animal models.

Since the current study is designed to assess the effects of spatial (i.e. cage versus aviary housing) and climatic (i.e. indoor versus outdoor) isolation, we expect that birds housed indoors in cages will experience a relative decrease in hippocampal neurogenesis

and hippocampal volume, whereas birds housed indoors in aviaries will only experience a relative decrease in neurogenesis. Further, we expect that birds housed outdoors in cages will experience a relative decrease (versus those housed in outdoor aviaries) in hippocampal volume, but not in neurogenesis. In line with the environmental harshness hypothesis, we expect that the relative decrease in neurogenesis of birds housed indoors will be greatest during the winter months.

Methods

Subjects and Housing

Thirty-two adult black-capped chickadees (*Poecile atricapillus*) were captured from the wild on University grounds and on private properties (with permission of the owner) in Halifax, NS, under a permit obtained from Canadian Wildlife Services (CWS; ST2779). We captured birds during three months of year between 2012-2014: February, May, and October, using potter traps or mist nets, depending on season. After capture, birds were kept in cloth bags for no longer than 1h before being transported to our facilities. Of these birds, 14 died, likely due to stress incurred during capture and transportation or during handling and injections (see below) .

Upon arrival to our facilities, birds were housed in one of four conditions: outdoor aviary (OA), indoor aviary (IA), outdoor cage (OC), or indoor cage (IC). Birds were assigned to housing conditions ad-hoc with consideration given to keeping group sizes balanced. In the OA group, birds were housed in individual 3.3 x 2.1 x 2.5 m outdoor aviaries consisting of metal alloy framing and wire mesh, located on the 4th floor roof of our departmental building. Each aviary was equipped with the following: a cylindrical birdhouse made of PVC tubing partially filled with beta chips; large tree branches; an

imitation Christmas tree (2m tall, 654 branch points); a plastic standing birdbath; and an assortment of food and water cups fixed to the Christmas tree branches and the sides of the aviary. Birds in the OC group were housed in individual 62 x 32 x 32cm cages positioned within an otherwise empty aviary on the roof. Each cage was fitted with a cylindrical birdhouse made of a PVC pipe (7.62 cm diameter) partially filled with beta chips, three perches, a small birdbath, and food and water cups fixed to the sides of the cage. The front of each aviary was covered with a camouflage-design hunting sheet to minimize visual stimuli caused by the researchers. A similar detached sheet was used to control for the same factor in the caged birds. Birds in the indoor groups were housed in aviaries and cages sporting identical setups to the outdoor housing units.

Indoor bird rooms were maintained on an updated light–dark cycle (updated weekly) closely resembling that of the natural light–dark cycle in Halifax, NS. Bird room temperature ranged from 19-21 ℃ regardless of time of year. All birds received ad libitum food and water. Diet consisted of ground sunflower seeds and Mazuri Small Bird Maintenance food, whole sunflower seeds, mealworms, and cuttle bone, replenished daily. Housing conditions and experimental procedures were approved by the Dalhousie University Committee on Laboratory Animals (UCLA) as outlined by the Canadian Council on Animal Care (CCAC).

Procedure

BrdU injections

Some tissue collected from these subjects was used to design an immunocytochemisty protocol for 5-bromo-2-deoxyuridine (BrdU) labeling. After a 24 h acclimation period, birds were given four 0.5 cc intramuscular injections of 1.5 mg/mL

BrdU in 0.1 M phosphate buffered solution (PBS; pH = 7.5) at 2 h intervals, as described in Hoshooley and Sherry (2004).

Behaviour Observations

To assess activity level, birds' behaviour was observed twice weekly between 08:00 and 09:00 hours, during which time chickadees are considered most active. Instantaneous observations of behaviour were recorded each minute for 0.5 h. Birds were scored as either moving, eating, drinking, preening, caching, or stationary.

Perfusion and Histology

Six weeks after BrdU injections, birds were killed via intraperitoneal overdose of Euthanyl and Xylazine solution (2.5 cc; 1:1). Birds were transcardially perfused with heparinized PBS until flushed (~3-5 min), followed by 4% paraformaldehyde (pH 8.5) until tissue became rigid (~3-5 min). Brains were harvested and kept in 4% paraformaldehyde for 24 h before being transferred to a 30% sucrose solution for cryoprotection. Once saturated, brains were frozen on pulverized dry-ice and stored at -80 °C until further processing. Using a cryostat (-16°C; 12° blade angle), brains were sectioned in the coronal plane at a thickness of 30 μm. Three series of tissue were collected at 120 μm intervals throughout the telencephalon and stored in cryoprotectant solution (30% sucrose and 30% ethylene glycol in buffer) at -20°C. Gonadectomies were performed on all birds for sex determination.

Doublecortin Immunohistochemistry

We used DCX-immunoreactivity (ir) as an estimate of hippocampal neurogenesis, following methods similar to those used by Wada et al. (2013) and Hall et al. (2014). For immunohistochemical processing, sections were transferred to phosphate buffered saline

(PBS) and washed twice. Sections were transferred to 0.5% H_2O_2 in dH_2O and incubated

for 30 m, and subsequently washed (2 x 5 m) in PBS. Sections were then incubated in

10% Normal Horse Serum (Vector Laboratories) in 0.3% Triton-X in PBS (Sigma;

PBS/T) for 1 h. Next, the sections were incubated in DCX primary goat antibody at 1:250

(C-18; Santa Cruz Biotechnology) overnight at 4°C. The following day, sections were

washed in 0.1% PBS/T and incubated with biotinylated horse anti-goat secondary

antibody (Vector Laboratories) for 1 h at room temperature. Sections were then washed

twice in 0.1% PBS/T and incubated in avidin-biotin horseradish peroxidase complex

(Vectastain ABC, Elite Kit) at 1:200 in 0.3% PBS-T for 1 h and subsequently washed

twice in 0.1% PBS-T. After another two washes in 0.1% PBS/T, tissue was reacted with

0.04% diaminobenzidine solution (Sigma FastDAB) for ~45 s to visualize antibody-

avidin-biotin complexes. Sections were rinsed four times in PBS, mounted on microscope

slides, dehydrated, cleared, and coveredslipped with permount.

Nissl Stain

One series of tissue was mounted on microscope slides for cresyl violet staining.

Slides were dipped in dH_20 to remove salt, and submerged in cresyl violet (Fisher) for 15

min. Slides were then transferred to glacial acetic acid in 70% ethanol for 30 s and

subsequently rinsed in 2 changes of 95% ethanol for 2 min each. After a 2 min rinse in

100% ethanol, slides were submerged in xylene for 10 min and coverslipped with

permount.

Imaging, Volumetric Analyses and Cell Counting

Images of DCX-ir sections were captured on the same microscope using a 40X

objective. Using similar methods to Wada et al. (2013) and Hall et al. (2014), we

measured the % of field of viewed covered by DCX-ir and the number of DCX+ cells in 5 sections of tissue at 120 μm intervals. The middle section was identified as having the largest cross-section area of HVC. We captured four sample squares in both hemispheres of each section of tissue: HA (control region), HVC, medial hippocampus (Hp1) and dorsomedial hippocampus (Hp2) (Figure 1). We used the threshold feature in ImageJ to estimate DCX-ir % cover in each image. For DXC+ cell counts, we exhaustively counted cells exhibiting features of fully differentiated neurons, including spherical or pyramidal shape, a densely stained cytoplasm, and protruding neurites (as described by Boseret et al., 2007). (Figure 2).

For the Nissl-stained sections, images of telencephalon were captured using an Epson Perfection 1650 flatbed scanner, while hyperpallium apicale (HA), HVC (not an acronym), and hippocampus (Hp) images were captured at 10X magnification using an Olympus BX51 microscope, Olympus U-TVO 5XC-3 camera, and Image Pro Plus 7.0 software. From Nissl stained images, area of HVC and Hp were calculated using ImageJ software by tracing the region's boundary in every mounted section (120 μm intervals) in which the region appeared (Figure 3). Area estimates for telencephalon, Hp and HVC were translated to volume using a formula for a truncated cone.

Results

Doublecortin labeling

We examined the number of DCx+ cells and the % coverage of DCX staining. For both dependent measures, I first performed an overall sex x location (in/out) x size (aviary/cage) x season (fall/winter/spring) x region mixed (HA/HVC/Hp1/Hp2) ANOVA however our sample size was too small to calculate interactions for most effects. For both

dependent measures, the only significant effect was of region (number of DCx+ cells: $F(3, 6) = 30.95$, $p < 0.001$); % coverage: $F(3,6) = 27.88$, $p = 0.001$). Post hoc tests for counts showed there were more DCx+ cells in Hp1 than in HA or Hp2. Post hoc tests for coverage showed there was more staining in HVC than in HA, and more staining in Hp1 than in all other areas. I then performed non-parametric tests separately for each region.

First, I compared counts and coverage in male (n=8) and female (n=4) birds for each region (Figure 4). Kruskal-Wallis tests showed there were no significant differences between sexes in DCX counts or % coverage for any region. We therefore could combine results for males and females in further analyses. To do this, we used appropriate tests (e.g. Mann-Whitney, Kruskall-Wallis) to analyse counts and coverage separately for each factor (location, size, season) on each region.

Figure 5 shows images of tissue sections stained with 3,3-diaminobenzidine for doublecortin from birds housed indoors and outdoors. Figure 6 (top panel) displays mean DCX+ cell counts separated by housing location (indoor/outdoor) for each region analyzed. The mean number of DCX+ cells in medial Hippocampus (Hp1) was higher in birds housed outdoors (n=6, M=218.03, SE=14.73) than birds housed indoors (n=6, M=141.25, SE=25.82), U = 18.50, p=0.041. There were no differences in the number of DCX+ cells between indoor and outdoor birds in any other region. Figure 6 (bottom panel) displays mean DCX % cover separated by housing location for each region analysed. There were no significant differences detected in any region; however, there was a trend of increased DCX % cover in Hp1 of birds housed indoors (M=13.47, SE=2.72) than in birds housed outdoors (M=3.44, SE=0.50), U = 29.00, p=0.093.

Figure 7 shows mean number of DCX+ cells (top panel) and % coverage (bottom panel) separated by size of housing (aviary/cage) for each region analysed. There were no differences between cage (n=4) and aviary (n=8) housed birds in any region for either measure.

Figure 8 shows images of tissue sections stained with 3,3-diaminobenzidine for doublecortin from birds captured during different seasons. Figure 9 displays differences in DCx+ cell counts (top panel) and % coverage (bottom panel) across season for each region. For the number of DCx+ cells, there was a significant difference among groups in Hp1 (U = 7.26, p = 0.026); winter birds had a higher number of DCx+ cells than spring or fall birds. No further effects were detected in DCx+ cells counts among seasons. For % coverage there was a significant effect of season in HVC (U = 6.68, p=0.36), Hp1 (U = 7.92, p=0.019), and Hp2 (U = 7.92, p=0.019). In HVC, there was greater coverage in fall than in spring but no difference between winter and fall or spring. In both Hp1 and Hp2, the most coverage was in winter, and there was significantly less in fall than in winter, and significantly less in spring than in fall.

Finally, DCx+ count and DCx % coverage were highly correlated in HVC (r(12)=0.925, p < 0.001), Hp1 (r(12)=0.76, p = 0.004), and Hp2 (r(12)=0.82, p = 0.001), but not in the control region HA (r(12)=0.29, p = 0.37). In addition, counts (r(12) = 0.62, p = 0.03) and coverage (r(12) = 0.83, p = 0.001) in Hp1 and Hp2 were correlated.

Volume

I first performed an overall sex x location (in/out) x size (aviary/cage) x season (fall/winter/spring) x region (Hp, HVC) mixed ANOVA; however, our sample size was too small to calculate interactions for all effects. I then conducted non-parametric tests

separately for each region, using region volume as a proportion of telencephalon. I tested differences between male (n=6) and female (n=3) birds for Hp (Figure 10, top left) and HVC (Figure 11, top left). As there were no significant differences between sexes in relative Hp and HVC volume, I combined male and female data. Figure 9 also shows relative Hp volume separately for season (top right), location of cage (bottom left) and size of cage (bottom right). I compared relative Hp volume across each factor using appropriate non-parametric tests. There were no significant differences detected among birds captured in fall (n=5), winter (n=2) or spring (n=2); between birds housed indoors (n=4) or outdoors (n=5); or between birds housed in cages (n=3) or aviaries (n=6). Figure 11 also shows relative HVC volume separately for season (top right), location of cage (bottom left) and size of cage (bottom right). I compared relative HVC volume across each factor using appropriate non-parametric tests. There were no significant differences among birds captured in fall (n=3), winter (n=1) or spring (n=2); between birds housed indoors (n=4) or outdoors (n=5); or between birds housed in cages (n=1) or aviaries (n=5).

Behaviour

Birds were observed for 4-6 sessions across the 6 weeks of the experiment. To equalize across birds and make data comparable across birds, we calculated the proportion of total observations that each behaviour occurred for each bird. We then averaged this proportion across birds of a particular group (e.g. indoor vs. outdoor). Some behaviours occurred extremely infrequently across all birds: caching (< 2%), eating (6.7%), drinking (<1%), and preening (<2%). I therefore only analysed activity by looking at the proportion of observations that were categorized as "moving" (37%) and

present these data in Figure 12. I first performed an overall sex x location (in/out) x size (aviary/cage) x season (fall/winter/spring) mixed ANOVA. There was a significant main effect of location, $F(1, 11)=48.66$. $p=0.02$; birds housed outdoors spent a greater proportion of their time moving than birds housed indoors (bottom left). I used non-parametric tests (i.e. Mann-Whitney, Kruskall-Wallis) to analyse differences in proportion of movement between sex (top left), size (bottom right) and season (top right) but these were not significant.

I performed correlation analyses on proportion of movement, DCX+ count, and DCX % cover. Proportion of movement was not significantly correlated with DCX+ cell counts in HA ($r(12) = 0.16$, $p = 0.61$), HVC ($r(12) = -0.25$, $p = 0.44$), Hp1 ($r(12) = 0.01$, $p = 0.98$) or Hp2 ($r(12) = 0.16$, $p = 0.34$). Proportion of movement was not significantly correlated with DCX % cover in HA ($r(12) = -0.48$, $p = 0.18$), HVC ($r(12) = -0.4$, $p = 0.20$), Hp1 ($r(12) = -0.22$, $p = 0.49$) or Hp2 ($r(12) = -0.36$, $p = 0.26$).

Discussion

In the current study, we sought to examine the effects of season, housing location and housing type on types on neurogenesis and volume of HVC and Hp in captive black-capped chickadees (*Poecile atricapillus*). We did find some seasonal change: in Hp, neurogenesis was highest in winter, while in HVC it was highest in fall. In addition, we showed that the location of housing was more important than size of cage: birds housed indoors had reduced neurogenesis compared to birds housed outdoors, however there were no differences in neurogenesis in birds housed in an aviary compared to a cage. Finally, volume of Hp and HVC were not affected by any factor. Taken together, our

results seem to indicate that ideal laboratory housing for studying neurogenesis in chickadees may be outdoors, but that cage size does not matter.

Seasonal Change in Hippocampal Neurogenesis

Previous results on seasonal changes in hippocampal neurogenesis and volume have been equivocal. Barnea and Nottebohm (1994), after finding a peak in hippocampal neurogenesis (i.e. survival of neurons born during the first day of captivity) during October, forwarded the idea that seasonal changes in neurogenesis coincide with onset of food-storing (i.e. hippocampus-dependent) behaviour, which begins as early as August and increases into the fall months (Sherry & Vaccarino, 1985). Our results, however, are more in line with those reported by Hoshooley, Phillmore, Sherry and MacDougall-Shackleton (2007): neurogenesis was highest during the winter months, following an increase between the spring and fall months. Our results cannot be explained by behaviour: we found no seasonal differences in birds' activity levels, and since our birds were fed ad libitum and not given opportunities to cache, it can be argued that the seasonal peak in hippocampal neurogenesis observed here is not directly associated with increased caching behaviour or reliance on spatial memory to retrieve food. This idea also fits with findings from Hoshooley, Phillmore, Sherry and MacDougall-Shackleton (2007): while birds in their study were given the opportunity to cache and retrieve food, amount of caching was not correlated with neurogenesis. In fact, birds in their study cached most during October. Thus, despite findings that hippocampal neurogenesis is modulated by memory use (Ladage, Roth, Fox, Pravosudov, 2009; Hall, Delaney & Sherry, 2014), seasonal changes of this measure probably rely, at least in part, on the presence of some external cue and not on caching behaviour itself.

Location of Housing

In the current study, chickadees housed indoors had reduced hippocampal neurogenesis (a 63% reduction) compared to those housed outdoors. This is interesting in that, as with our between-season comparisons, a reduction in hippocampal neurogenesis cannot be attributed to a change in caching and retrieval behaviour between these two housing conditions. In their study, Barnea and Nottebohm (1994) found that black-capped chickadees housed in large outdoor aviaries had a 50% reduction in hippocampal neuron survival compared to free-flying birds over a four to six week period. Birds in their outdoor aviary condition were permitted to store and retrieve seeds for two months prior to BrdU injections; thus, it can be inferred that the reduction in neurogenesis in these birds was not associated with an acute stressful event (i.e. acclimation to captivity). Further, it is not known whether the authors attempted to retain ecological validity in their aviaries by addition of tree branches and other items encountered in nature, or by limiting food access. Thus, it is difficult to predict how outdoor aviary conditions between my study and theirs may have differentially affected birds' brain and/or behaviour. Although we did not compare captive birds to free-flying birds, we were able to control for space and caching opportunity, allowing us to assess the impact of location and cage size independently from caching behaviour.

Interestingly, our finding that hippocampal neurogenesis is reduced in indoor birds does not replicate findings by Tarr, Rabinowitz, Imtiaz and Devoogd (2009), who report no difference in hippocampal neurogenesis between free-flying birds and birds housed indoors. One important difference between these two studies is that in their study, the authors delivered a single injection of BrdU to birds at the time of capture, meaning

that any BrdU+ cell identified after their 4-6 week housing period would have divided within approximately two hours of capture. During this time, birds likely experienced high level of stress, which has been shown to interfere with cell proliferation and cause apoptosis in other animal models (e.g. Heine, Maslam, Zareno, Joëls, & Lucassen, 2004). In the current study, DCX+ cells identified in hippocampi were likely born after approximately two weeks of housing, and therefore stress levels between groups would have been stabilized though acclimation to housing conditions. It is not known whether acute stress affects hippocampal neurogenesis in birds; however, rapid increases in plasma corticosterone leads to higher cache recovery rates in mountain chickadees (Saldanha, Schlinger & Clayton, 2000), whereas chronic treatment with corticosterone has no effect on hippocampal morphology and cell proliferation in black-capped chickadees (Pravosudov & Omanska, 2005).

Despite our control over caching behaviour in the current study, we found that birds housed indoors spent 10% less time moving than birds housed outdoors. This result is interesting because birds housed indoors also had reduced neurogenesis; thus, it could be argued that there was a relationship between motor activity and neurogenesis in these birds, which fits with studies in mammals showing that exercise can increase cell proliferation (e.g. van Praag, Kempermann & Gage (1999). Previously, changes in hippocampus were thought to coincide with onset of caching behaviour (Barnea & Nottebohm, 1994); however, subsequent studies found no such relationship (Hoshooley, Phillmore, Sherry, & MacDougall-Shackleton, 2007). It would be interesting to explore this relationship further; perhaps caching behaviour only correlates with hippocampal neurogenesis when it coincides with increased motor activity.

Although we strove to make many aspects of indoor and outdoor housing conditions consistent, there were still a number of environmental factors that varied between these conditions; for example, changes in weather, interaction with free-flying conspecifics, and a plethora of other visual and auditory stimuli not present in laboratories. On the other hand, laboratory housing presents its own set of stimuli, such as ambient noise, artificial lighting, and controlled temperature and humidity. So the particular variable, such as temperature, that may be responsible for the difference in neurogenesis between indoor and outdoor housing conditions may not be identifiable from our study, and may in fact be a combination of variables making up the climate in which birds were housed.

It could be argued from our findings that increasing the physical complexity (e.g. increased space, real and artificial tree branches) is not sufficient in maintaining levels that would presumably be present in free-flying birds. However, this argument raises the question of whether the reduction in hippocampal neurogenesis in indoor birds is detrimental. In many animals, survival during the winter months rests on a fine balance of immune function; thus, metabolic energy and access to food are invaluable resources (see Demas, 2004 for review). Since hippocampal neurogenesis is highest during the winter months (Hoshooley, Phillmore, Sherry, MacDougall-Shackleton, 2007), and since hippocampal neurogenesis is associated with environmental harshness (Chencellor, Roth, LaDage, & Pravosudov, 2010), it can be argued that it is adaptive. Therefore, it may be the case that reduction in hippocampal neurogenesis in birds housed indoors may effectively represent a bird's relief from unfavourable environmental conditions. Because

our outdoor birds had access to the same resources as indoor birds, if the aforementioned scenario is true, then temperature is a possible candidate as a perpetuating factor.

Chancellor, Roth, LaDage and Pravosudov (2010) found that hippocampal neurogenesis in black-capped chickadees increased as a function of environmental severity (as defined by factors including precipitation and temperature). Since we have shown that hippocampal neurogenesis can be reduced as a consequence of removal of natural stimuli (or presentation of unnatural stimuli), without manipulating behaviour of spatial confinement, then perhaps proximate cues from the environment should be considered as potential regulatory factors of neurogenesis. For example, in our study, indoor birds were maintained at the same ambient room temperature, regardless of season. It can be argued that the increase in temperature experienced by birds housed indoor is a potential proximate cue for decreased reliance on spatial memory (since during warm seasons, food resources are more readily available). While the effects of temperature on neurogenesis have not been directly studies in birds, it has been observed in reptiles (Penafiel, Rivera, Gutierrez, Trias, & De La Calle, 2001). Thus, future studies assessing a potential influence of ambient temperature on hippocampal neurogenesis will be valuable to further elucidate proximate cues influencing this seasonally variable process.

Size of Housing

In this study, cage size did not affect neurogenesis in chickadee hippocampus. Again we must consider the Barnea and Nottebohm (1994) results: they showed that chickadees held in an outdoor aviary had reduced hippocampal neurogenesis compared to free-flying chickadees. Their birds therefore were both housed outdoors, but one was in

captivity while the other was not. In our study, all birds were in captivity, and we varied size of housing. It is possible then, that birds in captivity, regardless of cage size, have reduced neurogenesis compared to free-flying birds. Said another way, allowing birds access to an aviary did not ameliorate effects of captivity.

Tarr, Rabinowitz, Imtiaz, and DeVoogd (2009) also assessed the effects of captivity on hippocampal neurogenesis. Our findings run contrary to theirs in that they found no reduction in hippocampal neurogenesis in indoor birds relative to their free-flying counterparts. However, in their study, the authors injected birds with BrdU at the time of capture and housed birds in outdoor cages for one night before being transferred indoors. It may be the case that since BrdU became incorporated into replicating DNA while birds were housed outdoors, cell proliferation was unaffected, and therefore the lack of an effect of indoor housing suggests that this variable does not affect cell survival. Since DCX is expressed in cells for less than 6 weeks, we can be confident that cells quantified in our indoors birds were born during the housing manipulation, rather than before. Therefore, it may be the case that cell proliferation, rather than survival, is affected by indoor housing.

Captivity and Hippocampal Volume

Our results are consistent with the majority of research on seasonal effects on hippocampal volume in black-capped chickadees: it does not change. (Hoshooley & Sherry, 2004; Hoshooley, Phillmore, Sherry, & MacDougall-Shackleton, 2006; but see Smulders, Sasson, & DeVoogd, 1995). Thus, it was not surprising that we did not detect significant differences in relative hippocampal volume across season. Further, we found no effect of captivity on hippocampal volume, which has been shown previously (Ladage

Roth, Fox, & Pravosudov 2009; Tarr & Devoogd, 2009). However, Ladage Roth, Fox and Pravosudov (2009) and Tarr, Rabinowitz, Imtiaz and Devoogd (2009) did find a 26% and 23% reduction in hippocampal volume between free-flying and captive chickadees, respectively. A major difference to note between our studies and the studies previously reporting reduced hippocampal volume in captive birds, is that birds in those studies were compared to free-flying counterparts, rather than cage versus aviary housing. It seems that the difference in space between aviary and cage housing is not sufficient to elicit a reduction in hippocampal volume. As we saw with neurogenesis, it may be the case that the change from free-living to captivity reaches a floor effect of reduction, meaning variance in the amount of spatial confinement does not matter. However, to determine whether this is the case, it would be beneficial to have a free-flying group in the current study to help to further disentangle the effects of housing condition on hippocampal neurogenesis and volume observed here. In addition, to confirm our free vs. captive hypothesis, we could house birds in an even larger aviary to see if that ameliorates the effects of captivity.

Effects of captivity on HVC

HVC is a nucleus in the song system of birds and is involved in song learning and production (see Nottebohm, 2005 for review). This structure undergoes changes according to breeding condition (see Dawson, King, Bentley, & Ball, 2001 for review). Despite consistent findings of increased volume in HVC in birds in breeding condition versus those in non-breeding condition, previous studies have failed to detect this change when using season as an independent variable (Phillmore, Hoshooley, Sherry, & MacDougall-Shackleton, 2006). Thus, it is not surprising that we found no effect of

season on this measure. To our knowledge, no studies have looked at the effects of captivity on HVC neurogenesis in this species. Previously, other factors such as nutritional stress during development and corticosterone treatment during adulthood negatively affect HVC volume and neurogenesis in other songbirds (Buchanan, Leitner, Spencer, Goldsmith, & Catchpole, 2004; Newman, MacDougall-Shackleton, Kriengwatana, & Soma, 2010). Here, we found that housing size, location, and season did not affect HVC neurogenesis. While a lack of an effect here may suggest that groups did not differ in stress level, given our small sample size, further research is needed to confirm this.

Measuring Neurogenesis: Methodological comparisons

Consideration of methodologies used to quantify neurogenesis lends insight to understanding conflicting findings. By using doublecortin to label neurogenesis, we quantified only those cells displaying characteristics of fully differentiated neurons; however, it is not known whether these neurons would have survived until maturity (because neurons stop expressing DCX before this time). Recall that we originally followed the methods of Barnea and Nottebohm (1994): we administered BrdU the day after capture, and killed birds after six weeks of captivity. This means we labeled dividing cells at the time of capture, and allowed sufficient time for cells born on the day of BrdU administration to become functionally integrated, mature neurons (Alvarez-Buylla & Nottebohm, 1988). However, because we ultimately used DCX to label the cells, it is possible that cells born at the time of capture during October had reached maturity by week 6, and thus were no longer expressing DCX. Therefore, our counts

would underestimate actual numbers of neurons in HP, and may have failed to detect effects of neuron survival between conditions.

Hoshooley, Phillmore, Sherry and MacDougall-Shackleton (2007) killed birds after 10 days in captivity, meaning that BrdU labeling was an estimate of neuronal recruitment to the hippocampus, rather than of neuronal survival. Considering this, it may be the case that, on average, neurons quantified in the current study were born two weeks later in the sampling period than would be the case for BrdU+ neurons in the same tissue. We are currently developing a BrdU+NeuN immunocytochemistry protocol that will allow us to make a more direct comparison of our results with those reported in previous studies, and allow us to determine how many BrdU+ cells born on the day after capture survive to 6 weeks. This protocol will also be important in assessing differences between BrdU and DCX labeling, especially in terms of various stages of neurogenesis –for example, recruitment versus survival. If DCX yields similar results as BrdU, then DCX may be a more convenient marker of neurogenesis, particularly when assessing neurogenesis in free-flying birds (see Balthazart & Ball, 2014 for a review of these methods).

Conclusions and future directions

We found that birds housed indoors had reduced neurogenesis compared to those housed outdoors, whereas cage versus aviary housing had no such effect. We also failed to detect a difference in hippocampal volume across any of our independent measures, supporting previous findings that annual changes in hippocampal volume are variable. Birds housed indoors also displayed reduced movement compared to those housed outdoors, suggesting that motor activity might play a role in mediating hippocampal

neurogenesis. However, if motor activity is involved, it is still not known what aspect of the environment either promotes or suppressed neurogenesis. Future studies may look at temperature as a potential factor mediating the relationship between indoor and outdoor housing on neurogenesis and should also measures subjects' stress response to determine whether any particular condition, or combination of conditions leads to increased stress and an ultimate reduction in neurogenesis.

Figure 1.
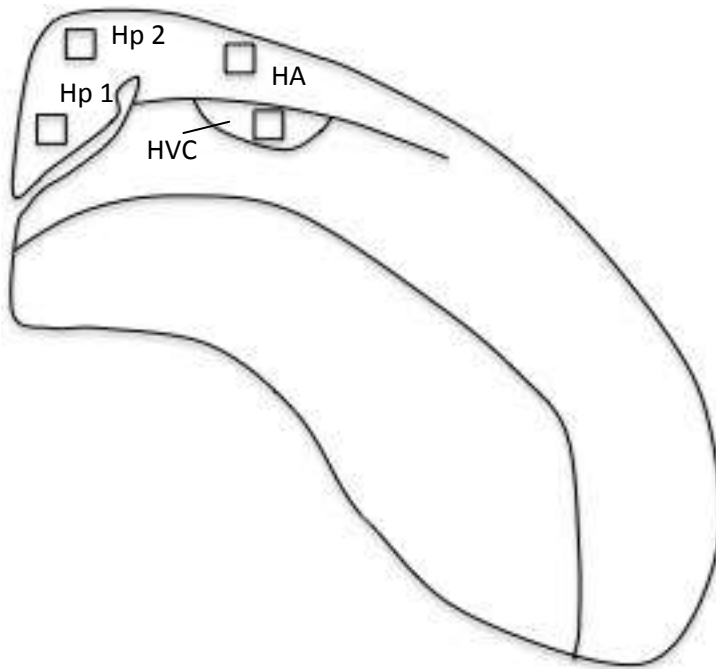
Schematic diagram of a coronal section of one half of the black-capped chickadee telencephalon. Four sampling squares were used to measure DCX-ir.
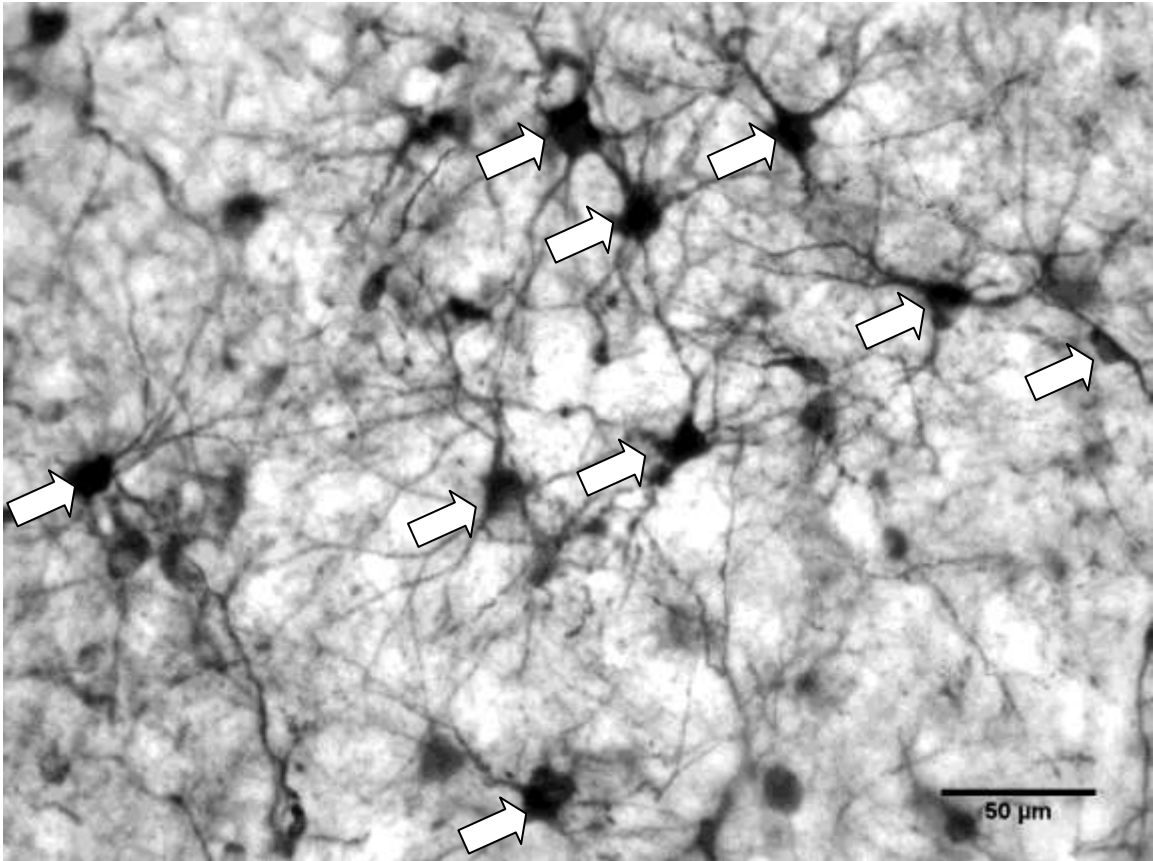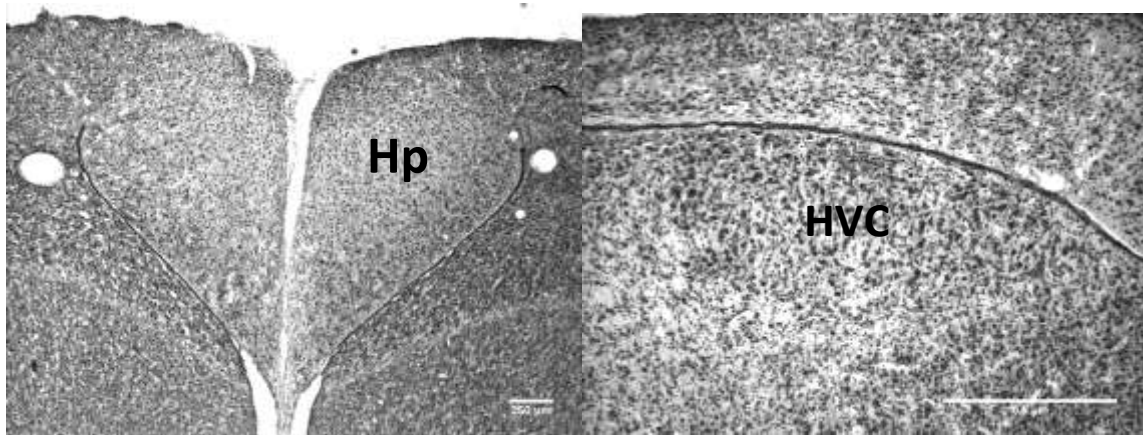
Figure 2.

Image of chickadee hippocampal section stained with 3,3 diaminobenzidine for the neuronal protein doublecortin (DCX) showing cells exhibiting neuronal characteristics (i.e. including spherical or pyramidal shape, a densely stained cytoplasm, and protruding neurites). Image was captured at 40X magnification.
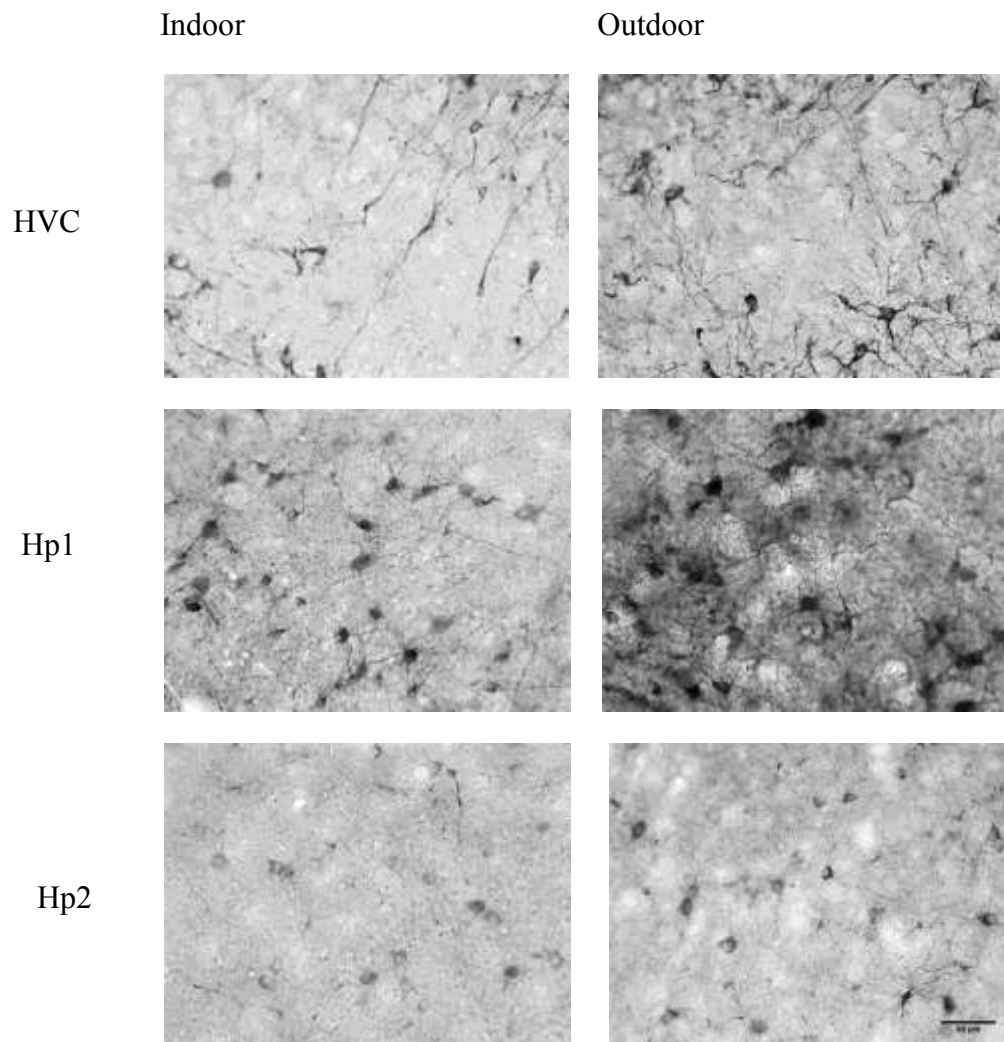
Figure 3.

Images of coronal sections of chickadee telencephalon stained with cresyl violet showing the hippocampus (left) and HVC (right). Images captured at 4X and 10X magnification, respectively.
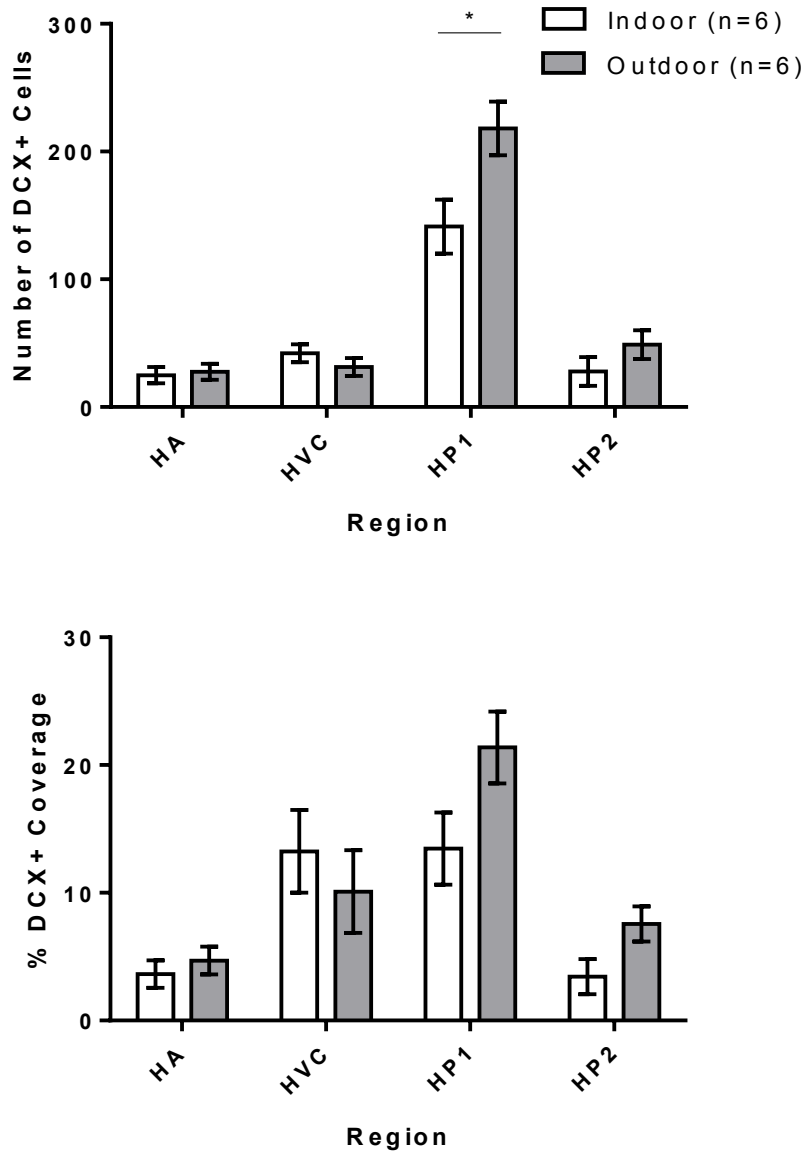
Figure 4.
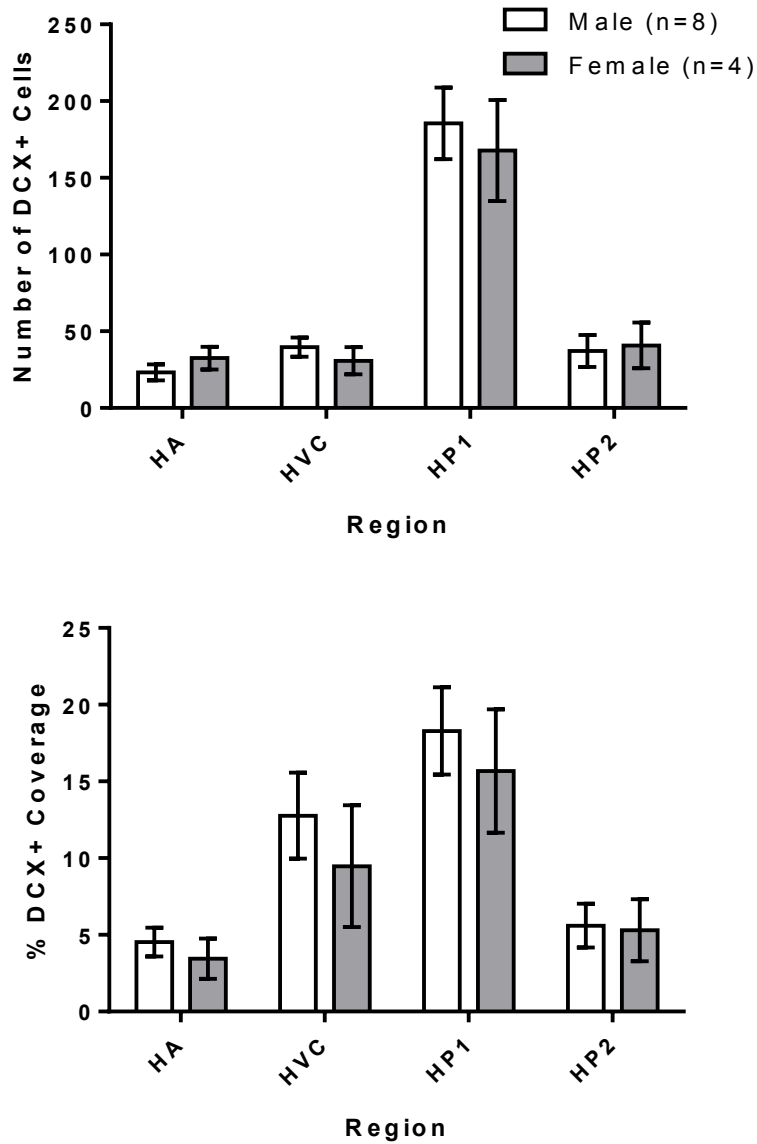
Average number of DCX+ cells (top) and average % DCX coverage (bottom) in all regions of interest (HA, HVC, Hp1 and Hp2) separated by sex of chickadee. Error bars represent standard errors of the means. There were no significant effects for any region.

Figure 5.

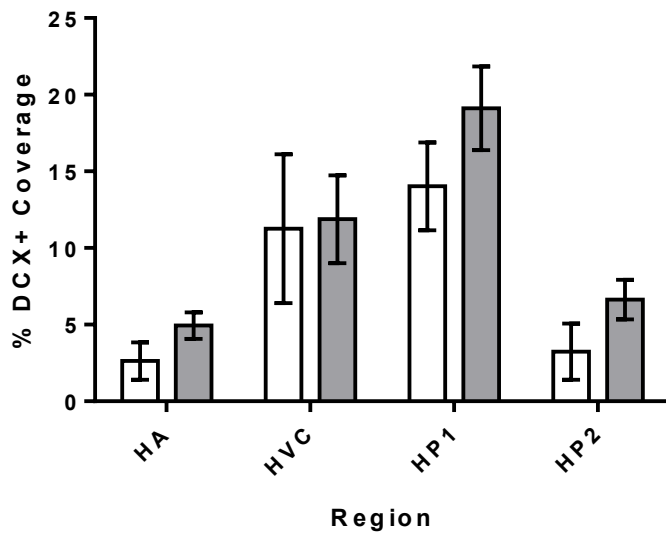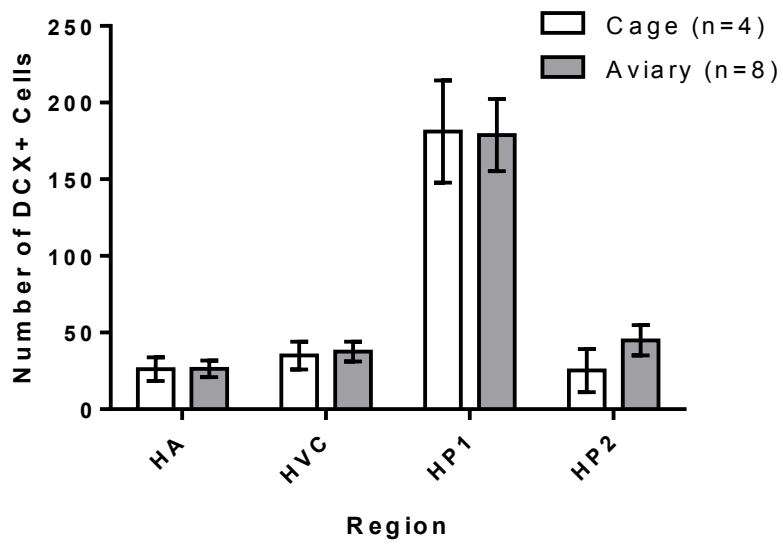Indoor   Outdoor

HVC

Hp1

Hp2

Images of sections stained with 3,3-diaminobenzidine for the neuronal protein
doublecortin (DCX) from HVC (top row), Hp1 (middle row) and Hp2). Each column
shows an example of a section from a bird housed indoors (left) or outdoors (right).
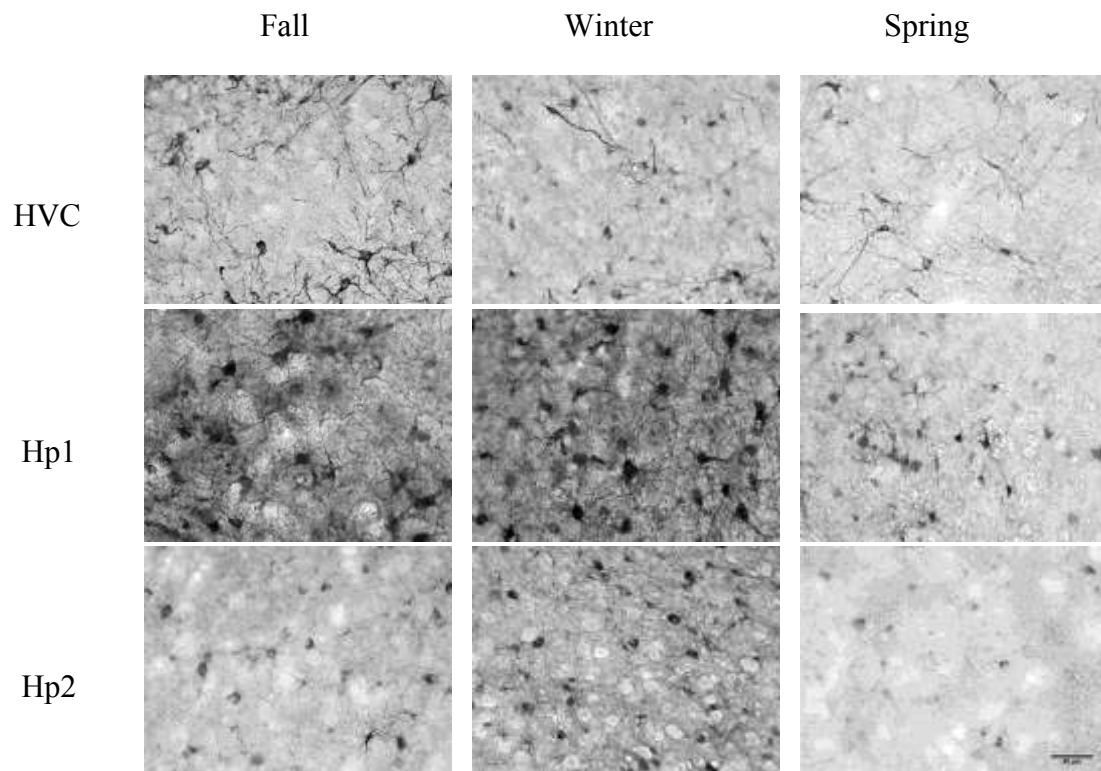
Figure 6.

Average number of DCX+ cells (top) and average % DCX coverage (bottom) in all regions of interest (HA, HVC, Hp1 and Hp2) separated by housing location. Error bars represent standard errors of the means. Bracket indicates a significant difference at $p < 0.05$.
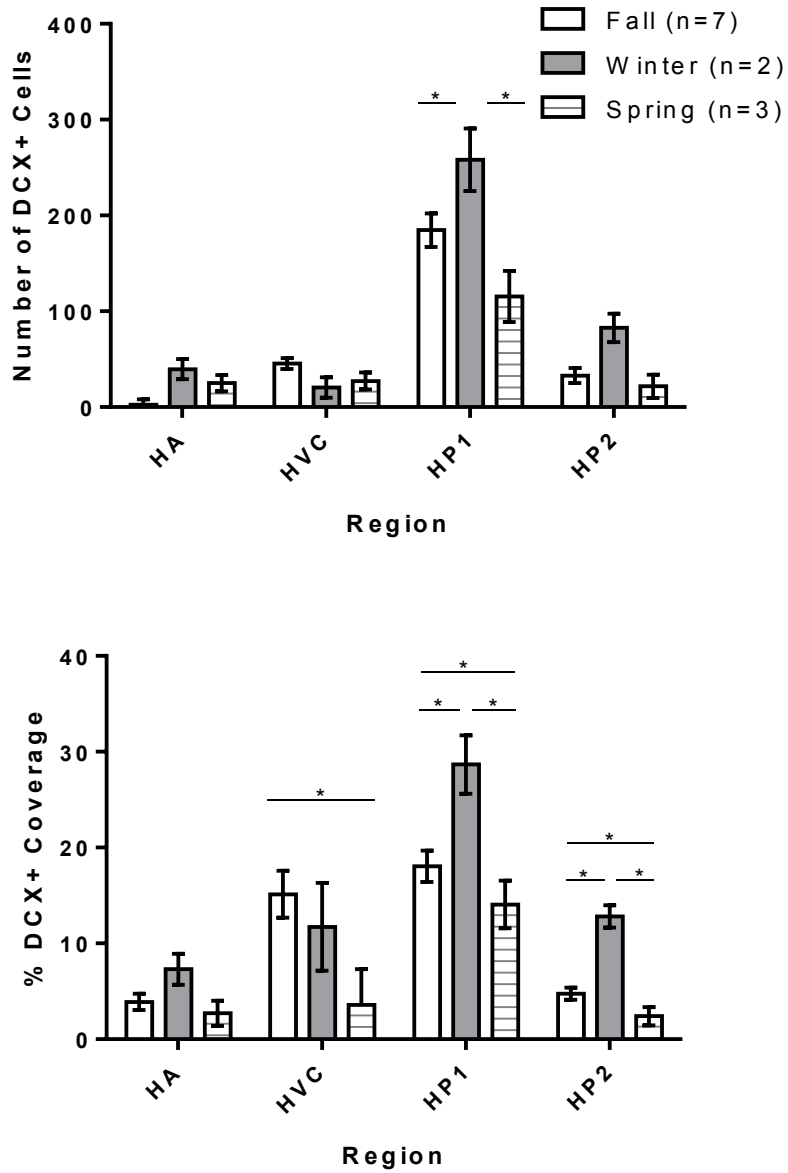
Figure 7.

Average number of DCX+ cells (top) and average % DCX coverage (bottom) in all regions of interest (HA, HVC, Hp1 and Hp2) separated by size of housing. Error bars represent standard errors of the means. There were no significant effects for any region.

Figure 8.

	Fall	Winter	Spring

HVC

Hp1

Hp2

Images of sections stained with 3,3-diaminobenzidine for the neuronal protein doublecortin (DCX) from HVC (top row), Hp1 (middle row) and Hp2). Each column shows an example of a section from a bird captured in fall (left), winter (middle) or spring (right).

Figure 9.

Average number of DCX+ cells (top) and average % DCX coverage (bottom) in all regions of interest (HA, HVC, Hp1 and Hp2) separated by season. Error bars represent standard errors of the means. Brackets indicate significant difference at P < 0.05.

Figure 10.
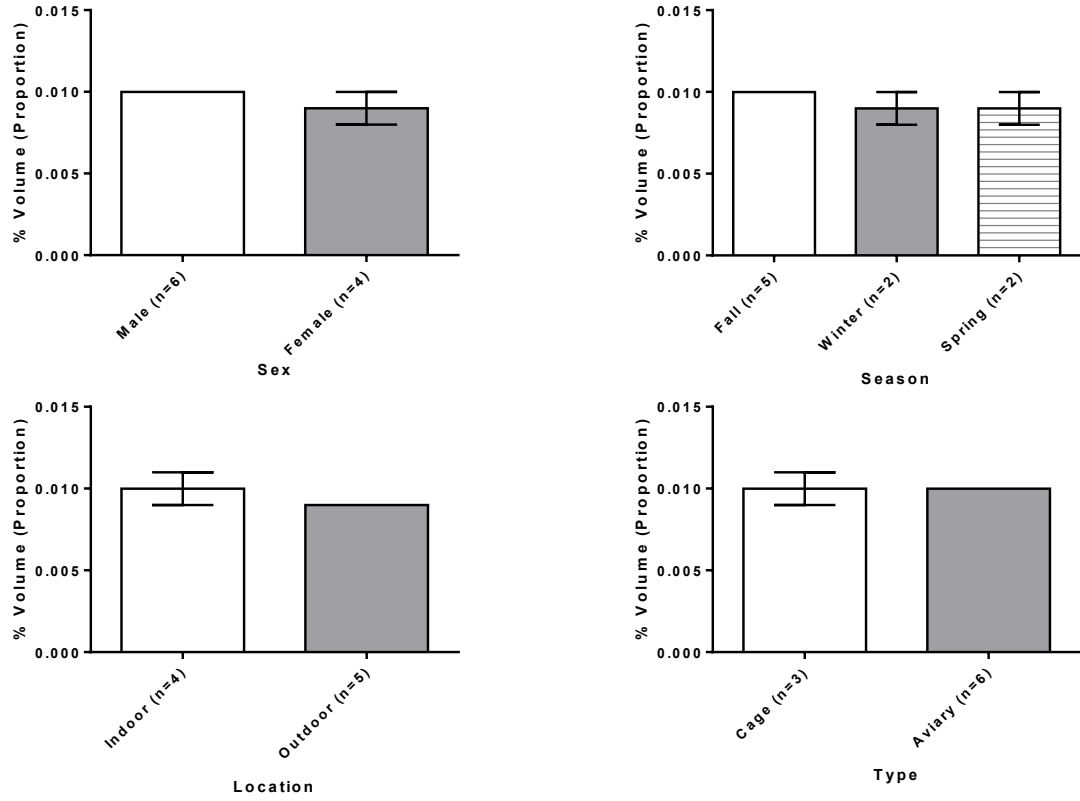
Average volumes of Hp in chickadees separated by sex (top left), season (top right), housing location (bottom left) and housing type (bottom right). Error bars represent standard errors of the means. There were no significant effects for any factor.

Figure 11.
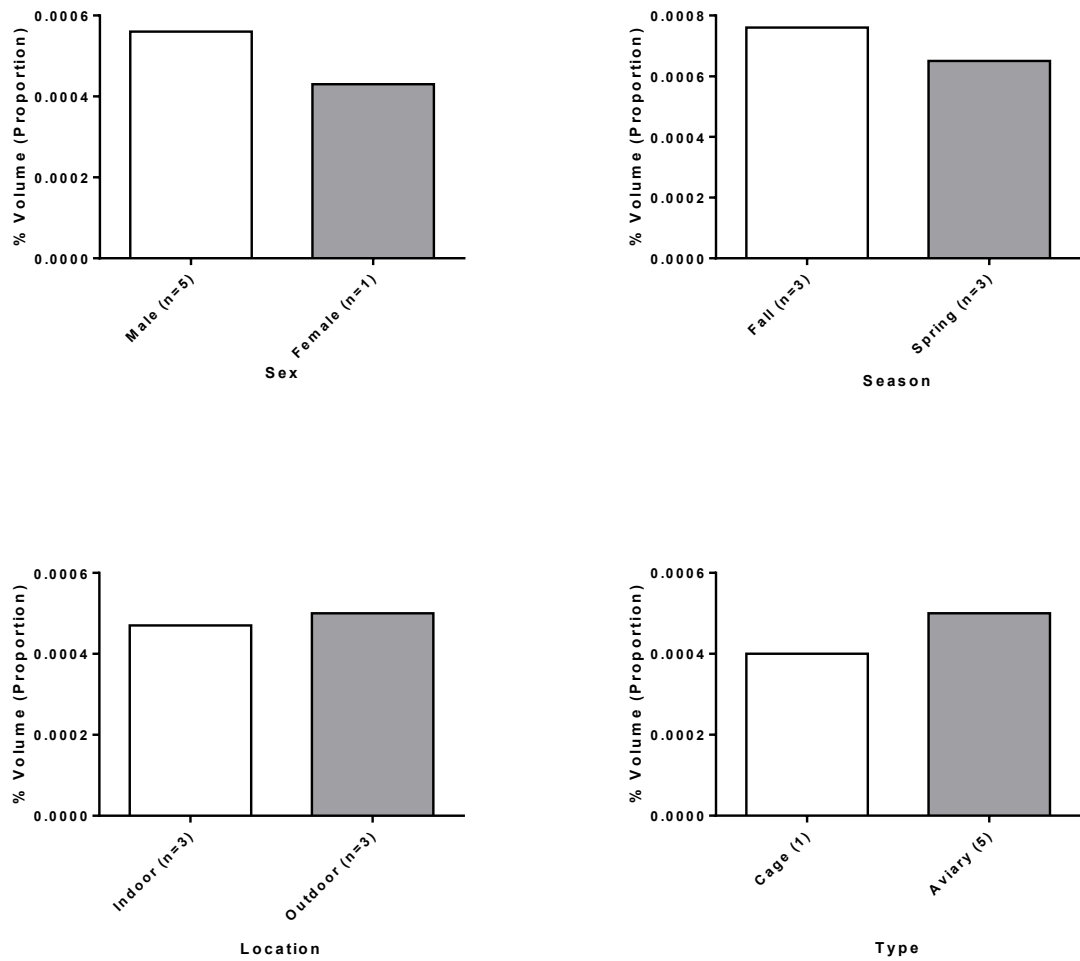
Average volumes of HVC in chickadees separated by sex (top left), season (top right), housing location (bottom left) and housing type (bottom right). There were no significant effects for any factor.

Figure 12.

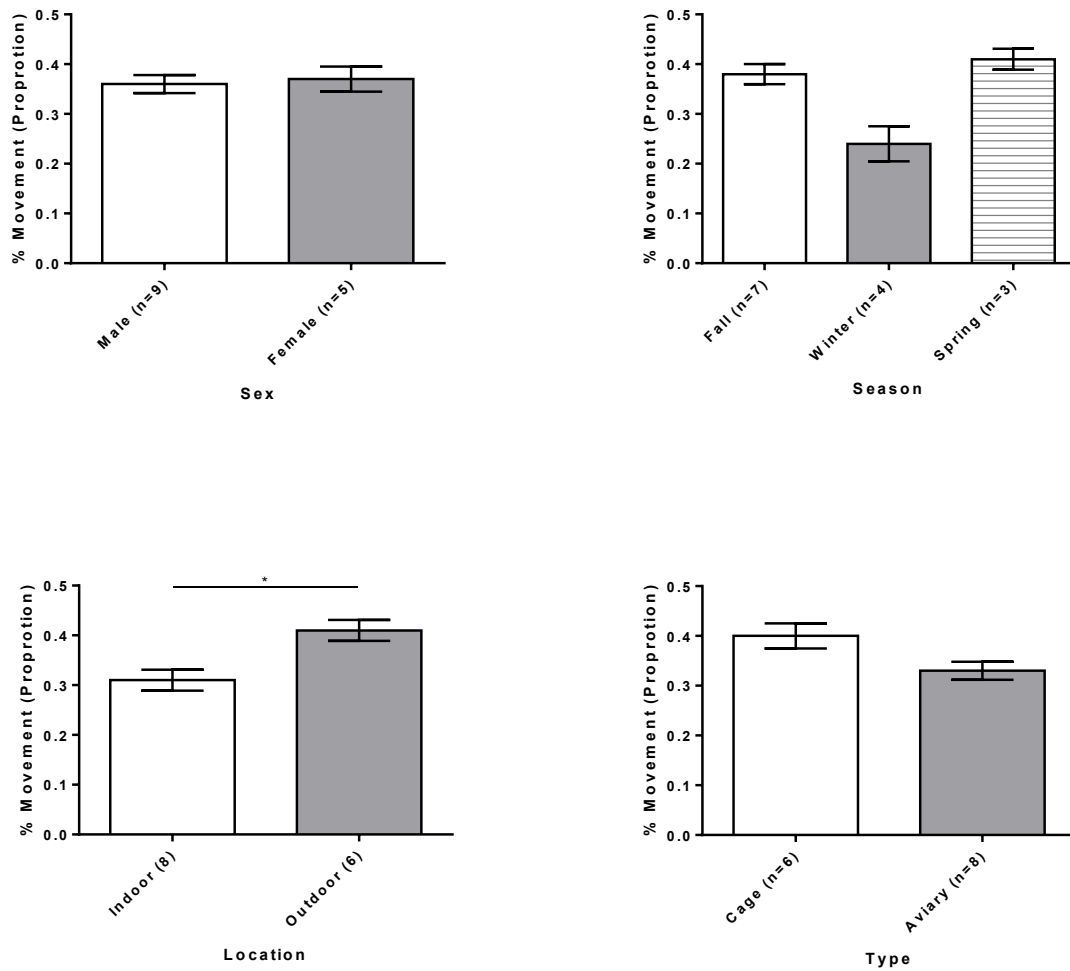Average proportion of observations in which chickadees were moving during the observation periods separated by sex (top left), season (top right), housing location (bottom left) and housing type (bottom right). Error bars represent standard errors of the means. Brackets indicate significant difference at $p < 0.05$.

Conclusion

In this study, we showed that housing location (i.e. indoor versus outdoor housing) seems to be a more powerful factor than housing type (i.e. cage versus aviary) in influencing hippocampal neurogenesis. Though I had natural elements from a birds environment as part of each housing condition (e.g. addition of real tree branches to all cages and aviaries), other factors associated with the natural environment may be necessary in maintaining neurogenesis at a level typical of black-capped chickadees in nature. One of these is temperature – both level and fluctuation. Manipulation of laboratory housing temperature to mimic the natural climate would be possible at some points of the year; however, this manipulation would be impossible during the winter months, when hippocampal neurogenesis seems to be highest. Alternatively, outdoor aviaries could be modified to be more like indoor housing by isolating birds from other factors of the natural environment, such as precipitation or visual stimuli while retaining the extreme temperatures occurring during the winter months. Testing this idea would give provide further insight to what proximate environmental factors might regulate neurogenesis, and provide opportunities for comparisons across class.

In terms of hippocampal neurogenesis, it can be argued from our findings that increasing the physical complexity (e.g. increased space, real and artificial tree branches) is not sufficient to maintaining level that would presumably be present in free-flying birds. However, this argument raises the question of whether the reduction in hippocampal neurogenesis in indoor birds is detrimental. A decline in resource availability may serve as a proximate cue for increased rates of neurogenesis; thus, it can be argued that a reduction in neurogenesis in birds housed indoor may represent a relief

from a harsh environment. Birds did not have to forage for food, compete with conspecifics, had access to shelter and water, and were protected from predators. With that is mind, research goals must be considered when deciding on animal housing. Is the goal to reduce stress and prevent mortality in captive animals, or is it to create the most ecologically valid experimental environment, so that results can be generalized to the animal in nature?

Other logical next steps in advancing this knowledge include adding a free-flying group to our study, and assessing captive birds' stress levels via plasma corticosterone. These additions will allow us to determine which housing condition gives rise to the hippocampal condition most closely resembling a free-flying bird's, as well as to gain insight as to whether stress is involved in regulating neurogenesis between various housing conditions. Further, it will be useful to attempt to increase the ecological validity of our outdoor aviary condition by making access to food more limited, as in the wild, and increasing opportunity to cache. Additionally, increasing the amount of activity birds do, especially in captivity, would help determine if activity levels are important for maintaining neurogenesis. This could be done with forced flying in a wind tunnel. In doing so, our aim would be to tease apart the effects of exercise from caching behaviour.

The lack of an effect of housing condition on hippocampal volume is consistent with other research, however we much consider that if there is seasonal change it is too small to detect with a between group design, and would appear if we looked at hippocampus in the same bird across season, or that variability is somewhat unpredictable (Hoshooley and Sherry, 2010). Given this, it may be useful for studies investigating structure volume in this model to consider alternative quantification

methods. Given that the avian hippocampus sits on the dorsal surface of the telencephalon and is lined by ventricles, this structure is particularly susceptible to damage during brain extraction from the skull, sectioning, and immunohistochemistry. It is not uncommon for this structure to become partially or fully disintegrated from adjacent regions during tissues processing. To preserve tissues integrity, or further, to acquire functional data from live birds, small animal MRI methods are likely to be used in future studies of seasonal plasticity in avian models. It will be interesting to see whether using these methods will help uncover the nature of changes in hippocampal volume.

A recently study demonstrated that exercise (i.e. flight in a wind tunnel) lead to increased hippocampal neurogenesis (among other regions) in European starlings (*Sturnus vulgaris*), but only in birds fed a low vitamin E diet (Hall et al., 2014). The authors suggest that since exercise and vitamin E can independently increase neurogenesis, that these factors are linked and context specific. Thus it could be argued that when food sources are scarce (i.e. during winter months), then neurogenesis is upregulated through exercise. This opens another interesting line of research that could be addressed with some straightforward modifications of our current experimental protocol.

Given the excitement that surrounds the field of neuroplasticity, and the potential for it to enhance human brains in ways previously thought impossible, it is convenient to accept the positive connotations that have become associated with the term. Indeed, a wide range of literature highlighting the exciting medical advances afforded by our knowledge of this phenomenon has become engrained in popular culture. In terms of

application to humans, studies of adult neurogenesis in animal models have been invaluable, and have guided experimentation. For example, implementing the human equivalent of a running wheel (i.e. participation in a regular exercise routine) can increase hippocampal volume, thereby reversing age-related decline in this measure (Erickson et al., 2011). However, with this desire to advance the field of neuroscience, it is important to keep in mind the adaptive value of this natural phenomenon, and to acknowledge that there could be situations in which less is more.

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