

Sex and Seasonal Variation in Hippocampal Volume and
Neurogenesis in the Eastern Chipmunk, *Tamias Striatus*

By

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ABSTRACT

The hippocampus (HPC) is important in spatial memory and navigation and also exhibits adult neurogenesis. In wild-living species, HPC volume and neurogenesis have been found to differ between the sexes and vary seasonally in tandem with spatial behaviours such as food-caching and mating. However, few studies have simultaneously compared across sex and season, and the literature contains inconsistencies. The present study examined sex and seasonal differences in HPC volume and neurogenesis in the eastern chipmunk, *Tamias Striatus*. HPC volume was greatest in males after controlling for age, consistent with males' greater spatial behaviour, but was seasonally stable. Neurogenesis exhibited a curvilinear pattern across the active season after controlling for age, with no sex or seasonal differences corresponding to the timing of spatial behaviours. The pattern of results was partially consistent with predictions based on chipmunk behavioural ecology, with some unexpected results, highlighting the importance of studies involving naturally variant populations.

Keywords: Hippocampus, Neurogenesis, Doublecortin, Neuroecology, Chipmunk

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TABLE OF CONTENTS

CHAPTER 1 - INTRODUCTION.....	1
THE HIPPOCAMPUS.....	2
NEUROECOLOGICAL STUDIES OF HIPPOCAMPAL VOLUME	4
Hippocampal Volume and Food-Caching Behaviour.....	5
Hippocampal Volume and Reproductive Behaviour	7
Sex and Seasonal Variation in Hippocampal Volume.....	10
Hippocampal Neurogenesis	13
Detection of Hippocampal Neurogenesis	14
Functional Role of Hippocampal Neurogenesis	15
Neuroecological Studies of Hippocampal Neurogenesis.....	17
Hippocampal Neurogenesis and Food-caching Behaviour.....	18
Sex and Seasonal Differences in Hippocampal Neurogenesis Related to Reproductive Behaviour.....	19
The Present Study	20
The Eastern Chipmunk	21
CHAPTER 2 - EXPERIMENT 1: SEX AND SEASONAL VARIATION IN HIPPOCAMPAL VOLUME	26
Introduction.....	26
Methods	29
Animals.....	29

Perfusions and Histology	31
Age Estimation	32
Hippocampal Volume Estimation.....	33
Statistical Analysis.....	33
Results.....	34
Age and Body Measurements	34
Sex and Seasonal Analysis of Body Measurements	34
Absolute HPC Volume	36
Controlling for Body Weight.....	37
Controlling for Lens Weight.....	39
Relative HPC volume	40
Controlling for Body Weight.....	40
Controlling for Lens Weight.....	41
Discussion.....	42
CHAPTER 3 - EXPERIMENT 2: SEX AND SEASONAL VARIATION IN	
HIPPOCAMPAL NEUROGENESIS	47
Introduction.....	47
Methods	50
Immunohistochemistry	51
Stereology	51
Statistical Analysis.....	52
Results.....	52

Absolute Estimates of DCX+ Cells 52

Controlling for Body Weight..... 53

Controlling for Lens Weight..... 55

Estimates of DCX+ Cells Relative to Total Granule Cells..... 55

Controlling for Body Weight..... 56

Controlling for Lens Weight..... 57

Granule Cell Estimates 58

Dentate Gyrus Volume 60

Discussion 61

CHAPTER 4 - GENERAL DISCUSSION..... 66

CONCLUSIONS 75

REFERENCES 76

LIST OF TABLES

Table 1. 29

Table 2. 35

LIST OF FIGURES

Figure 1. Map of Peterborough, ON showing the locations where chipmunks were caught.
..... 31

Figure 2. A) Representative sections showing the chipmunk HPC from anterior (top) to posterior (bottom). B) A representative chipmunk brain (anterior at top, posterior at bottom).....	37
Figure 3. Mean (\pm SEM) absolute volumes of the HPC in male and female chipmunks. Displayed means are corrected for body weight.....	38
Figure 4. Mean (\pm SEM) absolute volumes of the HPC in male and female chipmunks. Displayed means are corrected for lens weight	39
Figure 5. Mean (\pm SEM) proportions (%) of the HPC to surrounding tissue in males and females. Displayed means are corrected for body weight.	41
Figure 6. Mean (\pm SEM) proportions (%) of the HPC to surrounding tissue in males and females. Displayed means are corrected for lens weight.....	42
Figure 7. Photomicrograph of the chipmunk DG showing DCX-positive cells (red) and counterstained with DAPI (blue).	53
Figure 8. Mean estimates of DCX-positive cells (\pm SEM). Displayed means are corrected for body weight.....	54
Figure 9. Mean estimates of DCX-positive cells (\pm SEM). Displayed means are corrected for lens weight	55
Figure 10. Mean (\pm SEM) numbers of DCX-positive cells relative to the total number of granule cells. Displayed means are corrected for body weight	57
Figure 11. Mean (\pm SEM) numbers of DCX-positive cells relative to the total number of granule cells. Displayed means are corrected for lens weight.....	58
Figure 12. Mean (\pm SEM) number of total granule cells in males and females.	59
Figure 13. Mean (\pm SEM) volume of the DG in males and females.	61

Figure 14. Mean (\pm SEM) absolute volumes of the HPC in male and female chipmunks. Displayed means are not corrected for age covariates.....	88
Figure 15. Mean (\pm SEM) proportions (%) of the HPC to surrounding tissue in males and females. Displayed means are not corrected for age	89
Figure 16. Mean estimates of DCX-positive cells (\pm SEM). Displayed means are uncorrected for age covariates..	90
Figure 17. Mean (\pm SEM) numbers of DCX-positive cells relative to the total number of granule cells. Displayed means are not corrected for age covariates.	91

LIST OF APPENDICES

APPENDIX 1.....	88
Supplementary Figures	88

LIST OF ABBREVIATIONS

ANOVA	Analysis of variance
ANCOVA	Analysis of covariance
BrdU	Bromodeoxyuridine
CA(1-3)	Cornu ammonis (subfields 1-3)
DCX	Doublecortin
DG	Dentate gyrus
dP4	Deciduous premolar
HPC	Hippocampus

M3	Third molar
PBS	Phosphate-buffered saline
PCNA	Proliferating cell nuclear antigen
pP4	Permanent premolar
PSA-NCAM	Polysialated neural cell adhesion molecule

CHAPTER 1 - INTRODUCTION

The idea that adaptive phenotypes accumulate within populations over time via evolution by natural and sexual selection is a fundamental paradigm in biology. The probability that an organism will successfully reproduce is affected not only by its physical characteristics, but also by its behavioural traits. Certain behaviours increase the likelihood of survival and reproduction whereas others may be maladaptive or neutral. Hence, behavioural traits that are relevant to reproduction and survival should undergo selection pressure, and the most adaptive behaviours should accrue within a population over successive generations. Given that the nervous system is the proximate cause of behaviour, it is assumed that adaptive behaviours result from corresponding neurophysiological adaptations, however subtle or minute. Neuroecology, a subfield of neurobiology is an attempt to delineate the relationship between the brain and evolutionarily-derived behaviours in natural populations (Sherry, 2006).

The neuroecological approach is an important complement to laboratory-based research in understanding the nervous system. Studies in naturally-occurring populations may reveal the evolutionary significance of neurophysiological phenomena and their relevance to ecologically-relevant behaviours (Boonstra, Galea, Matthews, & Wojtowicz, 2001; Roth, Brodin, Smulders, LaDage, & Pravosudov, 2010). Although laboratory-based behavioural testing is absolutely essential for determining causal links between brain and behaviour, the immersion and isolation of the organism in a highly synthetic environment may not capture the full spectrum of behaviour or neural activity of the organism under conditions that the organism is specifically adapted to thrive under. Additionally, an awareness of a given brain region or behaviour's evolutionary advantages, tradeoffs, and

constraints is critical for drawing comparisons among species or between a model organism and humans (Roth et al., 2010).

This thesis focuses on the application of the neuroecological approach to understanding the relationship between spatial behaviour and the hippocampus (HPC), a brain area critical for spatial memory (Squire, Stark, & Clark, 2004; Sutherland, Sparks, & Lehmann, 2010) and a site that exhibits adult neurogenesis, or the birth of new neurons in adulthood (Amrein & Lipp, 2009). I will review the literature regarding the function of the HPC and hippocampal neurogenesis as well as the studies that have examined how the HPC and neurogenesis differ in a range of wild-living species according to the spatial behaviours these species perform in the wild. I will then describe the present experiment, which examines sex and seasonal differences in HPC volume (Chapter 1) and neurogenesis (Chapter 2) in wild-living eastern chipmunks.

THE HIPPOCAMPUS

The hippocampus (HPC) is a structure present in most mammalian and avian species. In mammals, the HPC is conventionally defined as consisting of the cornu ammonis (subfields CA1-3) and the dentate gyrus (DG), with some variation in definitions that confine the HPC proper to only the cornu ammonis as well as some that include the subiculum (Amaral & Lavenex, 2007; Squire et al., 2004). The avian HPC occupies the dorsomedial cortex and lacks the mammalian cornu ammonis and dentate gyrus (Colombo & Broadbent, 2000; Székely, 1999). Although the mammalian and avian HPC appear structurally different, there is ample evidence that they are homologous in both embryonic development and cognitive function (Colombo & Broadbent, 2000). Thus, from functional and anatomical perspectives, the HPC is a homologous structure in multiple species.

Multiple lines of evidence indicate that the HPC is crucial for spatial and non-spatial long-term memory. Amnesia following damage to the HPC has been described in several human patients who exhibit impairments recalling autobiographical memories and past events (Rempel-Clower, Zola, Squire, & Amaral, 1996; Scoville & Milner, 2000), a range of recognition memory tests for words and pictures (Reed & Squire, 1997; Rempel-Clower et al., 1996; Scoville & Milner, 2000; Zola-Morgan, Squire, & Amaral, 1986), and spatial memories such as path finding (Maguire, Nannery, & Spiers, 2006) and odor-place associations (Goodrich-Hunsaker, Gilbert, & Hopkins, 2009). Lesions to the HPC in non-human animals impair long-term memory in a number of behavioural tasks such as contextual fear conditioning (Lehmann, Lacanilao, & Sutherland, 2007; Maren, Aharonov, & Fanselow, 1997), object recognition (Broadbent, Squire, & Clark, 2004; Gaskin, Tremblay, & Mumby, 2003; Mahut, Zola-Morgan, & Moss, 1982), and various tests of spatial memory and navigation (Clark, Broadbent, & Squire, 2005; Morris, Garrud, Rawlins, & O'Keefe, 1982; Watanabe & Bischof, 2004).

Further evidence of the involvement of the HPC in long-term spatial and non-spatial memory comes from imaging studies. In humans, the intact HPC shows greater activity during mental navigation along memorized paths (Ghaem et al., 1997), recalling spatial and non-spatial relations between memorized pictures (Ryan, Lin, Ketcham, & Nadel, 2010), and recalling spatial and non-spatial information about past events (Hoscheidt, Nadel, Payne, & Ryan, 2010). Similarly, studies of immediate early gene expression in rodents and birds reveal increased activity of HPC neurons when animals perform non-spatial memory tasks including contextual fear conditioning (Hall, Thomas, & Everitt, 2001), and socially-transmitted food preference (Ross & Eichenbaum, 2006), and

various spatial memory tasks (Guzowski, Setlow, Wagner, & McGaugh, 2001; Mayer, Watanabe, & Bischof, 2010).

Moreover, strong evidence of the role of the HPC in spatial memory and navigation comes from the discovery of place cells, which become preferentially active in response to an animal visiting different locations (Moser, Kropff, & Moser, 2008; O'Keefe & Dostrovsky, 1971). The finding that the HPC directly encodes the location of an animal in the environment led to the formulation of cognitive map theory (O'Keefe & Nadel, 1978), several different versions of which have since emerged (Jacobs & Schenk, 2003). Cognitive map theories generally hold that the HPC plays a role in generating a mental representation of space and memory for locations (Jacobs & Schenk, 2003). Other theories de-emphasize the role of the HPC in spatial representation *per se* and propose more general views of HPC function that include the binding of multiple elements in a learning episode (Rudy & Sutherland, 1995) or the representation of spatial and temporal relations between events (Eichenbaum, Dudchenko, Wood, Shapiro, & Tanila, 1999). Nonetheless, the HPC appears to be a critical structure for spatial memory and navigation.

NEUROECOLOGICAL STUDIES OF HIPPOCAMPAL VOLUME

Neuroecology makes the general prediction that differences in the behavioural ecology of wild-living species requiring differential cognitive capacity will manifest as differences in the anatomy or physiology of brain areas subserving the particular cognitive functions necessary in performing such behaviours. Of relevance to this thesis is the principle of proper mass, which predicts that the size of the brain region is positively correlated with its role in behaviour (Jerison, 1975). Given the metabolic cost of neural tissue (Foley, Lee, Widdowson, Knight, & Jonxis, 1991), enlargement of a brain area

without a corresponding increase in cognitive capacity would be maladaptive (Jacobs, 1996). Thus, when a neural region and its associated behaviour(s) enhances the survival of a specific species or improves a mating opportunity, we would predict that this species would exhibit a greater regional neural volume than species that do not accrue the same benefit (Roth et al., 2010).

With respect to the HPC, this theory suggests that species that rely extensively on spatially complex behaviours for survival or enhanced mating opportunity would be expected to have a larger HPC than species that rely less on spatial memory. Studies of the HPC in wild mammals and birds have generally found this to be the case (Jacobs, 1996; Sherry, Jacobs, & Gaulin, 1992; Sherry, 2006).

Hippocampal Volume and Food-Caching Behaviour

Many birds and mammals feed by storing caches of food that they later return to in order to feed, rather than consuming food where and when it is first found. Bird species that cache food in the wild exhibit better spatial memory (Brodbeck, 1994; Pravosudov & Clayton, 2002), food-caching experience increases HPC volume in laboratory-raised birds (Clayton & Krebs, 1994) and damage to the HPC impairs the successful retrieval of food caches (Sherry & Vaccarino, 1989; Watanabe & Bischof, 2004). These findings indicate that food-caching species have evolved better spatial memory and that the HPC mediates successful food-caching behaviour. Thus, the neuroecological prediction that follows is that greater food-caching behaviour should be correlated with greater HPC volume across wild-living species.

The correlation between food-caching behaviour and HPC volume was first established by two landmark studies of food-storing and non-food-storing passerine birds.

Sherry, Vaccarino, Buckenham, & Herz (1989) quantified the HPC volumes of 23 species of passerine birds and found that, relative to telencephalon volume, food-caching species had a larger HPC than non-food-caching species. Krebs, Sherry, Healy, Perry, & Vaccarino (1989) found similar results in a study of 32 species of passerine birds in which the HPC volume of birds that cached food in multiple locations was larger than in birds that did not engage in food-caching. Additionally, Healy & Krebs (1992) found that in seven species of corvid, HPC size was correlated with the amount of food-caching behaviour engaged in by each species, strengthening the view that HPC size varies with the extent of food-caching behaviour. Thus, it appears that in birds, more spatially-complex food-caching behaviour is correlated with a larger HPC.

The relationship between HPC volume and food-caching has received relatively less attention in mammals than in birds. However, the extant studies support the prediction that HPC volume and food-caching are correlated. In mammals, food caching behaviour can be roughly categorized into two types: Scatter-hoarding, where animals cache food in multiple locations; and larder-hoarding, where animals cache all their food in a central hoard (Brodin, 2010). Both food-caching strategies presumably require long-term/spatial memory capacity either to remember the location and contents of scattered food caches, or to remember food sources from which to harvest for the building of larder-hoards. The research on mammals has tended to examine differences in HPC volume as it relates to the type of hoarding that species exhibit, rather than whether related species hoard or not.

Jacobs & Spencer (1994) compared the HPC volumes of 3 species of kangaroo rat. Mirriam's kangaroo rats, which engage in scatter-hoarding, had the largest HPC volume while Bannertail kangaroo rats, which defend a single food larder, had the smallest HPC.

Ord's kangaroo rats, a species with intermediate spatial complexity of feeding behaviour relative to the other two species, accordingly had intermediate HPC volumes. Additionally, Johnson, Boonstra, & Wojtowicz (2010) compared the HPC volumes of two populations of North American red squirrels. Eastern red squirrels engage in scatter-hoarding, whereas western red squirrels engage in larder-hoarding, which does not require the memorization of multiple cache locations. Although overall HPC volume was not measured, the authors compared the volumes of individual subfields of the HPC and found that eastern red squirrels had a larger dentate gyrus. These findings indicate that in mammals, HPC volume, or at least the volume of a principle subfield, correlates with the type of food-caching behaviour. Further, these data support the position that scatter-hoarding is more spatially complex than larder-hoarding.

Overall, the prediction that HPC volume would be correlated with food-caching behaviour has been supported by studies of a number of wild-living species. This relationship has been most thoroughly investigated in avian species. However, the evidence from wild-living rodents, albeit scant, also tends to support the view that greater spatial complexity in food-caching is associated with greater HPC volume, or augmentation of the volume of specific HPC subfields.

Hippocampal Volume and Reproductive Behaviour

Many species exhibit sexual dimorphism in spatial memory and the primary evolutionary driver of such sex differences is arguably differences in reproductive strategy in many cases (Jacobs, 1996; Jones, Braithwaite, & Healy, 2003). Successful reproduction often involves increased spatial behaviour one sex, such as increased home range size during breeding in polygynous rodents (Bowers & Carr, 1992; Gaulin & FitzGerald, 1988;

Thompson, 1978) or brood-parasitism in cowbirds (Rothstein, Yokel, & Fleischer, 1987). These evolved patterns of sexually-dimorphic spatial behaviour are also mirrored by corresponding differences in spatial memory performance (Galea, Kavaliers, & Ossenkopp, 1996; Guigueno, Snow, Macdougall-Shackleton, & Sherry, 2014). Given the sex differences in spatial behaviour and memory associated with certain breeding systems, HPC volume should be sexually-dimorphic in species with breeding behaviours are more spatially complex, with the sex engaging in the most spatially-complex reproductive behaviour exhibiting a larger HPC.

Accordingly, sex differences in HPC volume have been found in several wild species with sexually dimorphic spatial behaviour during mating. Jacobs et al. (1990) found that in polygynous meadow voles, males have a larger HPC than females, owing to the fact that male meadow voles increase their home range size during breeding (Gaulin & FitzGerald, 1988), and thus have greater spatial memory demands. In contrast, they found that monogamous pine voles do not exhibit a sex difference in HPC volume, as pine voles do not exhibit a sex difference in range size during breeding. Additionally, Jacobs & Spencer (1994) found that in both Mirriam's and Bannertail kangaroo rats, two polygynous species in which males also increase their range size during breeding (Behrends, Daly, & Wilson, 1986a; Randall, 1991), males had a larger HPC than females. These two studies provide evidence in support of the prediction that sex differences in HPC volume should occur in species with sexually-dimorphic spatial behaviour during breeding.

Sex differences in HPC volume have also been observed in brood-parasitic cowbirds. Many species of cowbirds use other bird's nests to lay their eggs, brood parasitism; however there are species differences related to this behaviour. Shiny cowbirds

are brood parasites in which females seek out and lay eggs in the nests of other birds, a spatially-complex behaviour that the males do not assist in. Both males and female screaming cowbirds seek nests in which to lay eggs. Bay-winged cowbirds, in contrast, are not brood-parasitic, so neither sex engages in this behaviour. Thus, the spatial memory requirement for cowbird reproduction may have species specific biases toward females rather than males in certain species, as these female cowbirds lay their eggs in the nests of other birds and must locate and monitor suitable nest sites (Rothstein et al., 1987). And indeed, these female cowbirds have better spatial memory than males (Guigueno et al., 2014). Reboreda, Clayton, & Kacelnik (1996) examined the HPC of three different species of cowbird. The authors found that the HPC was not only larger in parasitic versus non-parasitic cowbirds, but that female shiny cowbirds had a larger HPC than the males. Sherry, Forbes, Khurgel, & Ivy (1993) found similar results when they examined sex differences in HPC volume in brood-parasitic brown-headed cowbirds as well as closely related but non-parasitic red-winged blackbirds and common grackles. Female brown-headed cowbirds had a larger HPC than males, whereas there were no sex differences in the other two species. These results provide further confirmation of the prediction that sex differences in the spatial complexity of reproductive behaviour should be accompanied by sex differences in HPC volume.

Overall, the relationship between HPC volume and the spatial complexity of reproductive systems appears strong within the extant literature. In species where one sex engages in more space use for breeding, HPC volume appears consistently higher in that sex. These findings further support the general prediction that HPC volume is related to the degree of spatial behaviour performed in the wild.

Sex and Seasonal Variation in Hippocampal Volume

There is evidence that the HPC does not exhibit static volumes year-round. In several mammalian and avian species, the HPC actively changes volume in conjunction with changes in spatial behaviour related to mating, food-caching, and hibernation (see Yaskin, 2011 for review). Accordingly, laboratory studies that simulate seasonal change by manipulating photoperiod length have found both sex and seasonal differences in spatial learning performance (Galea, Kavaliers, & Ossenkopp, 1994; Pyter, Reader, & Nelson, 2005; Walton et al., 2011). Such seasonal change in the HPC within individuals may be an adaptive response to reduce the metabolic cost of HPC tissue during periods when there is less demand on spatial memory (Jacobs, 1996). Seasonal variation in the HPC may also interact with sex differences in species that exhibit seasonal and sexual dimorphism in spatial behaviour such that one sex may undergo seasonal change in the HPC while the other sex remains static.

The general theoretical prediction that follows from this is that in wild-living species with sexually- and seasonally-variable spatial behaviour, HPC volume should increase during the season containing the highest degree of spatial behaviour. This seasonal increase in HPC volume should also be greatest in the sex that increases its spatial behaviour the most. Indeed, one study fits this pattern exactly. Clayton, Reboreda, & Kacelnik (1997) compared the HPC across sex and season in two species of cowbirds. Shiny cowbirds, in which females (but not males) search for nests to parasitize, displayed a sex-specific seasonal change in the HPC, with only females having a larger HPC during the breeding season. Screaming cowbirds, which are brood-parasitic with both males and females participating in locating candidate nests, exhibited a seasonal change in HPC

volume in which both males and females had a larger HPC during the breeding season. Thus, HPC volume in cowbirds appears to closely track sex and seasonal changes in spatial behaviour in accordance with the above prediction.

Sex and seasonal variation in HPC volume has also been studied in wild-living rodents as well. Burger, Saucier, Iwaniuk, & Saucier (2013) examined HPC volume in Richardson's ground squirrels. Although the authors found a significant sex by season interaction, the timing of the sex difference was opposite to the predicted pattern, with males having a larger HPC during the non-breeding season and no sex difference during breeding. The authors note that, although polygynous, males of this species may not rely significantly on increased spatial memory during breeding because females remain concentrated in colonies. Additionally, only male Richardson's ground squirrels hoard food in the fall. Thus the HPC of this species may relate more to selection pressures around food-hoarding behaviours than to those involving mating behaviours.

Lavenex, Steele, & Jacobs (2000) investigated the HPC volumes of eastern gray squirrels during food-caching in October as well as both breeding seasons in January and June, when males substantially increase their range size. Thus, males should show larger HPC in January and June than during other periods. Although males had a larger HPC than females, no seasonal variation was observed in either sex. As eastern gray squirrels are relatively long-lived rodents, Lavenex et al. (2000) argue that their study represents a true test of seasonal HPC variation in adult animals, whereas other studies that examined short-lived animals may have simply detected sex-dependent developmental effects of a number of factors on HPC morphology.

In both mammals and birds, the evidence regarding seasonal change in the HPC is variable and inconclusive. Although sex and seasonal variation in HPC volume has been found in some cases (Burger et al., 2013; Clayton et al., 1997), other studies have failed to find this effect (Hoshooley & Sherry, 2004; Lavenex et al., 2000a). Additionally, the pattern of sex and seasonal variation in HPC volume is not consistently found within the same species (Hoshooley, Phillmore, Sherry, & Macdougall-Shackleton, 2007; Hoshooley & Sherry, 2004; Smulders, Sasson, & DeVoogd, 1995). Even when sex or seasonal variation in HPC volume is observed, the pattern of HPC volume changes is sometimes contrary to the pattern predicted from the behavioural ecology of the species in question (Burger et al., 2013; Hoshooley & Sherry, 2007).

Several factors may be responsible for the lack of consistency between such experiments. For one, the timing of adaptive change in HPC volume may be tied to the intensity of food-caching behaviour, which can peak at variable times of year and vary between years (Pravosudov, 2006). Because the volume of the HPC does not appear to be directly affected by changes in seasonal cues, such as changes in photoperiod (Krebs, Clayton, Hampton, & Shettleworth, 1995; but see Pyter et al., 2005), it has been suggested that in the wild, conflicting results regarding volume changes in the HPC may be tied to natural variation in the intensity of food-caching (Sherry & Hoshooley, 2009). In some species, it is also possible that the spatial demands of one behaviour, such as food-hoarding, may outweigh the spatial demands of mating, leading to seasonal increases in HPC at unpredicted times of year (Burger et al., 2013). Additionally, confounds related to age and lifespan, in which age differences between samples, could affect a cross-seasonal analysis (Clayton et al., 1997). Further, differences in the seasonal stability of the HPC may

exist between long- and short-lived animals (Lavenex et al., 2000). Thus, seasonal comparisons of the HPC may be confounded by nuances of spatial behaviour that are specific to particular species that vary from year to year alongside differences among age and lifespan. Broad assumptions about behavioural ecology such as "range size increases during mating equals greater spatial behaviour" may lack the necessary subtlety to make meaningful connections between seasonal changes in the HPC and spatial behaviour (Roth et al., 2010).

Hippocampal Neurogenesis

Having reviewed some of the relevant literature concerning how gross variation in hippocampal morphology maps onto neuroecological predictions regarding the relationship between spatial behaviours in the wild and their neural substrates, the focus of this review will now shift to neurogenesis (the birth of new neurons) in the HPC. The HPC is one of the few areas of the brain that exhibits neurogenesis during adulthood. Progenitor cells in the subgranular zone (SGZ) of the DG undergo mitosis and differentiate into both neurons and glia (Cameron, Woolley, McEwen, & Gould, 1993). The dividing cells that become neurons undergo several developmental stages from birth to maturity that are often categorized into proliferation (the production of new cells from the mitosis of progenitor cells) and survival (the maturation and integration of adult-born granule cells into the network of the DG) (Gage, Kempermann, Palmer, Peterson, & Ray, 1998; Lehmann, Butz, & Teuchert-Noodt, 2005). Surviving adult-born granule cells migrate from the SGZ into the granular layer of the DG (Cameron et al., 1993) and quickly extend processes to form synaptic connections with CA3 neurons (Hastings & Gould, 1999).

Detection of Hippocampal Neurogenesis

Newly born cells in the DG can be detected through a variety of methods. Thymidine analogues such as Bromodeoxyuridine (BrdU), a particularly popular marker used in many neurogenesis studies, can be administered to living animals that are incorporated into the DNA of newly-divided cells (Balthazart & Ball, 2014; von Bohlen und Halbach, 2011). BrdU is advantageous in that it allows determination of the age of newborn cells because all labelled cells can only have divided after BrdU administration and can track adult-born granule cells well into maturity as it remains in cell nuclei for months provided no additional cell divisions take place, which dilutes the cellular concentration of BrdU (Balthazart & Ball, 2014). However, there are several disadvantages to the use of BrdU. The bioavailability of BrdU can differ between species and physiological conditions, leading to differences in the number of labelled cells after a given BrdU dose (Balthazart & Ball, 2014). Moreover, additional assays are required to discriminate between newly-born neurons and other DNA synthesis events such as the birth of glia (Cameron et al., 1993; von Bohlen und Halbach, 2011). When detecting neurogenesis in wild-caught animals, the use of BrdU requires that animals are captured, injected with BrdU, and housed in captivity for several days to allow incorporation of the marker into dividing cells. The stress of capture and subsequent captivity can cause sufficient stress on wild-living animals to affect rates of neurogenesis, confounding the analysis of 'natural' rates of neurogenesis (Chawana et al., 2014).

For the purposes of detecting neurogenesis in wild-caught animals, labelling endogenous cellular markers of immature neurons may be preferable over exogenous markers like BrdU for the above reasons. Several endogenous markers of neurogenesis

have been discovered such as Ki-67 (a marker of ribosomal RNA transcription, Bullwinkel et al., 2006), PCNA (associated with DNA polymerase A, Mandyam, Harburg, & Eisch, 2007), and PSA-NCAM (polysialated neural cell adhesion molecule, Bonfanti, 2006). One particularly popular endogenous marker for neurogenesis, however, is doublecortin (DCX) (Balthazart & Ball, 2014; von Bohlen und Halbach, 2011). DCX plays a role in stabilizing cytoskeletal microtubules during neuronal differentiation and migration (Francis et al., 1999; Moores et al., 2006). It is expressed during a window of a few weeks after cell division and subsides as markers for mature neurons begins to be expressed (Brown et al., 2003). Colocalization with BrdU labelling reveals that 60-90% of BrdU-labelled cells in the DG express DCX (Couillard-Després et al., 2005; Rao & Shetty, 2004) and nearly all DCX-positive cells also expression endogenous, neuron-specific markers (Rao & Shetty, 2004). Moreover, DCX-positive cells do not coexpress markers for glia (Couillard-Després et al., 2005; Rao & Shetty, 2004). The *DCX* gene is also conserved across a variety of species (Reiner et al., 2006). Given these findings, DCX labelling appears to be an effective method to selectively detect immature neurons in the DG of many species without the need for pre-administration of any exogenous compounds.

Functional Role of Hippocampal Neurogenesis

Several factors affect both the proliferation and survival of adult-born granule cells, either increasing or decreasing neurogenesis. Differences in baseline rates of neurogenesis are observed in various species (Epp, Scott, & Galea, 2011; Klaus & Amrein, 2012). Within species and individuals, neurogenesis is reduced by stress hormones (Brummelte & Galea, 2010; Gould, McEwen, Tanapat, Galea, & Fuchs, 1997; Wong & Herbert, 2006) and is negatively correlated with age, steadily decreasing over the lifespan (Amrein, Isler,

& Lipp, 2011; Amrein, Slomianka, Poletaeva, Bologova, & Lipp, 2004; Barker, Wojtowicz, & Boonstra, 2005; Kuhn, Dickinson-Anson, & Gage, 1996). Neurogenesis is also increased by exercise (van Praag, Kempermann, & Gage, 1999) and sexual experience (Leuner, Glasper, & Gould, 2010). Gonadal hormones also have a strong effect on neurogenesis (Galea, 2008) with estrogen decreasing neurogenesis in females (Galea & McEwen, 1999; Ormerod & Galea, 2001) and testosterone increasing neurogenesis in males (Ormerod & Galea, 2003). Thus a number of factors affect neurogenesis in the individual.

Although the functional role of hippocampal neurogenesis has not yet been fully clarified, there is broad consensus that it is somehow important in HPC-dependant learning and memory (Marín-Burgin & Schinder, 2012; Wojtowicz, Askew, & Winocur, 2008). Studies that abolish neurogenesis report impairments in several HPC-dependent memory tasks (Jessberger et al., 2009; Saxe et al., 2006; Snyder, Hong, McDonald, & Wojtowicz, 2005; Winocur, Wojtowicz, Sekeres, Snyder, & Wang, 2006). Newly-born granule cells may play a role in spatial memory soon after division, as they are active (Chow, Epp, Lieblich, Barha, & Galea, 2012; Kee, Teixeira, Wang, & Frankland, 2007) and exhibit plastic changes in their dendritic arbor (Tronel et al., 2010). Several studies have also found that rates of neurogenesis increase in response to spatial learning (Ambrogini et al., 2000; Epp, Haack, & Galea, 2010; Epp et al., 2011; Gould, Beylin, Tanapat, Reeves, & Shors, 1999; Keith, Priester, Ferguson, Salling, & Hancock, 2008). These findings provide substantial evidence that hippocampal neurogenesis plays a functional role in spatial learning.

However, some studies fail to find a correlation between spatial learning and neurogenesis (Merrill, Karim, Darraq, Chiba, & Tuszynski, 2003; Van der Borght, Wallinga, Luiten, Eggen, & Van der Zee, 2005) and some studies find that learning may reduce neurogenesis (Ambrogini et al., 2004; Dagyte et al., 2009; Pham, McEwen, Ledoux, & Nader, 2005). Thus, the exact role of neurogenesis in HPC function and memory is not entirely clear, and additional research is required to clarify this issue. Moreover, few studies have examined neurogenesis in wild-living animals; such studies may provide more information about the evolutionary significance of neurogenesis.

Neuroecological Studies of Hippocampal Neurogenesis

From a neuroecological perspective, fewer studies have examined hippocampal neurogenesis than HPC volume, which may be problematic (Roth et al., 2010). However, cross-species comparisons suggest that rates of neurogenesis differ across species (Amrein et al., 2004; Barker, Boonstra, & Wojtowicz, 2011; Epp et al., 2011; Snyder et al., 2009), and may even be absent in some (Amrein, Dechmann, Winter, & Lipp, 2007; Patzke et al., 2013). Further, studies of neurogenesis in wild-living species suggest that these species may even respond differently to learning (Epp et al., 2011). Investigations of neurogenesis in wild species may be particularly insightful as environmental enrichment increases neurogenesis in laboratory animals (Kempermann, Kuhn, & Gage, 1997) and presumably wild-living organisms have more complex environments than can be achieved in laboratory settings. Indeed, some have suggested that standard laboratory conditions may not fully stimulate the brain's full capacity for neurogenesis (Boonstra, Galea, et al., 2001). Thus, studies of neurogenesis will provide important and novel insights in the understanding of functional and evolutionary significance of the HPC.

Similar to the logic of studies examining changes in HPC volume, rates of neurogenesis may relate to the degree to which a given species or individual engages in behaviour requiring spatial memory, given the apparent role of neurogenesis in spatial memory. However, hypotheses regarding the relationship between spatial behaviour and neurogenesis in natural populations must also account for reproductive status and age.

Hippocampal Neurogenesis and Food-caching Behaviour

Successfully remembering the location of food sources as well as the locations of cached food requires spatial memory depends on hippocampal neurogenesis (LaDage, Roth, Fox, & Pravosudov, 2010; Pan et al., 2013; Snyder et al., 2005; Winocur et al., 2006). Thus, species that engage in caching, e.g. wild black-capped chickadees, have higher rates of neurogenesis than non-caching species, house sparrows (Hoshooley & Sherry, 2007). Similarly, Barker, Wojtowicz, & Boonstra (2005) found that eastern grey squirrels, which hoard food in multiple sites, had higher neurogenesis than yellow pine chipmunks, which hoard their food in a single larder. Thus, there appears to be differences in neurogenesis between species that correlate with differences in food-caching behaviour.

However, baseline rates of neurogenesis vary among species (Amrein et al., 2004; Epp et al., 2011). As such, differences in neurogenesis between species engaging in different degrees of food-caching must be interpreted with caution. When comparisons of neurogenesis are made between two closely-related species or within the same species, neurogenesis does not appear to be correlated with food-caching (e.g. Johnson et al., 2010). For instance, comparisons between scatter-hoarding east-coast red squirrels and larder-hoarding west-coast red squirrels found no difference in neurogenesis, despite the difference in spatial complexity between these two caching strategies. Additionally,

Hoshooley & Sherry (2004) found that in black-capped chickadees, rates of neurogenesis did not vary across seasons, despite the fact that food-caching behaviour was greatest during the fall. These results are an indication that in the wild, food-caching alone is not highly predictive of neurogenesis rates, and that other factors influencing neurogenesis may be at play.

Sex and Seasonal Differences in Hippocampal Neurogenesis Related to Reproductive Behaviour

Sex and seasonal variation in reproductive behaviours may also correlate with changes in neurogenesis in natural populations. As discussed earlier, males of polygynous commonly increase their range size during the breeding season, while females keep their range size constant (Behrends et al., 1986a; Behrends, Daly, & Wilson, 1986b; Elliott, 1978; Gaulin & FitzGerald, 1988; Randall, 1991). This increase in ranging should lead to increased spatial cognitive demand and as a result, more neurogenesis, specifically in males during breeding.

Only three studies have examined both sex and seasonal differences in neurogenesis simultaneously in natural populations, and there is significant variation in the results of these studies. Galea and McEwen (1999) found that in wild meadow voles, neurogenesis was higher in non-breeding females than in males or breeding females. In contrast, Burger et al. (2014) found that in Richardson's ground squirrels, neurogenesis was higher during the non-breeding than the breeding season as it was in Galea and McEwen (1999), although males had higher neurogenesis than females, regardless of season. Note that Galea and McEwen (1999) did not observe a sex difference in voles. Contrary to both these studies, Lavenex et al. (2000) found no sex or seasonal differences in the eastern gray squirrel.

Notably, not one of these studies found evidence of neurogenesis increasing in males during breeding, the period with the greatest spatial cognitive demand. Some authors attribute neurogenesis fluctuations in wild-living rodents to steroid hormone fluctuations (Burger et al., 2014; Galea & McEwen, 1999). Neurogenesis is greatly affected by gonadal hormone fluctuations across breeding conditions (Galea, 2008). Estradiol reduces neurogenesis in females (Galea & McEwen, 1999; Ormerod & Galea, 2001), whereas testosterone enhances neurogenesis in males (Ormerod & Galea, 2003). Additionally, neurogenesis is suppressed by stress hormones (Brummelte & Galea, 2010; Gould et al., 1997) which peak during the breeding season in some wild-living populations of mammals (Boonstra, Hubbs, Lacey, & McColl, 2001; Eggermann, Theuerkauf, Pirga, Milanowski, & Gula, 2013). However, drawing such conclusions raises the question of whether neurogenesis is responding to steroid hormone fluctuations, or to changing cognitive demands, as would be predicted by neuroecologists.

The Present Study

More research is needed to clarify the relationship between sex, season, and changes in the HPC in mammals. The body of literature on the HPC in wild species to date contains a number of irregularities and variability in results. Previous studies contain possible confounding variables such as sexual dimorphism in food-caching (Burger et al., 2014, 2013), differences in lifespan (Lavenex et al., 2000a), and possible after effects of hibernation on the HPC during the spring breeding season (Popov et al., 2007; Popov, Kraev, Ignat'ev, & Stewart, 2011; Weltzin, Zhao, Drew, & Bucci, 2006). Selecting a species for analysis whose behavioural ecology is more amenable to avoiding these confounds may provide a clearer picture of the correlation between HPC anatomy and

natural behaviour. Furthermore, the examination of more species is needed in order to create a broader evolutionary picture of sex and seasonal effects on the HPC (Barker et al., 2011).

Additionally, age related differences between subjects have been overlooked in some studies (Clayton et al., 1997; Pan et al., 2013), whereas other studies have accounted for age using discrete variables such as scarring on males from mating competition (Burger et al., 2014, 2013), tooth colour (Burger et al., 2014, 2013), or the presence of adult molars (Smith & Smith, 1972). The absolute age of wild-caught animals cannot be determined without knowing their birth dates, but continuous variables that give an estimate of relative age may be more helpful in elucidating finer age-related differences between subjects. Body weight is positively correlated with age (Smith & Smith, 1972) and can act as a continuous age variable, but it can also be confounded by an over- or -underabundance of food from individual to individual and by emergence from hibernation (Panuska, 1959). The weight of the eye lens is also positively correlated with age (Augusteyn, 2014; Cavegn et al., 2013; Epp, Barker, & Galea, 2009; Hardy, Quy, & Huson, 1983) and is presumably not significantly affected by food availability. Combined, eye lens weight and body weight could provide an estimate of relative age differences on a continuous scale and would provide a useful control measure to account for potentially confounding variables.

The Eastern Chipmunk

The eastern chipmunk, *Tamias Striatus*, is one ideal species in which to examine sex and seasonal differences in the HPC. Eastern chipmunks are small sciurid rodents native to eastern North America (Snyder, 1982). They exhibit little or no sexual dimorphism in food-caching (Elliott, 1978); as such, potential confounds related to sex

differences in caching are removed unlike studies involving Richardson's ground squirrels. Chipmunks engage in two breeding seasons, allowing the examination of HPC volume during a breeding season that is distal to the end of hibernation, removing another potential confound (Elliott, 1978; Pidduck & Falls, 1973; Smith & Smith, 1972). Finally, no other study, to the author's knowledge, has conducted such an investigation of wild-living eastern chipmunks, and studies investigating additional species are needed to provide a broader evolutionary picture of the relationship between the HPC and natural behaviours (Barker et al., 2011).

Previous work has examined the relationship between the spatial complexity of natural behaviour and differences in the brain in several species of chipmunks. Budeau and Verts (1986) examined the relationship between habitat structural complexity and cranial volume in four different species of *Eutamias*, a chipmunk genus closely related to *Tamias*. They found that cranial volume was positively correlated with the structural complexity of the species' respective habitats, suggesting that life in a more spatially-complex environment could be associated with increased brain size. Additionally, Pan et al. (2013) found that the intensity of scatter-hoarding behaviour was related to neurogenesis in Siberian chipmunks. Thus, evidence from related species suggests that variation in the brains of eastern chipmunks can be expected, and that these changes would be related to differences in natural behaviour.

Habitat. Chipmunks tend to reside in deciduous woodlands (Snyder, 1982). They are solitary, living alone in complex burrow systems located at the center of a home range that can occupy between ~ 1.5 and ~ 3 km² (Elliott, 1978; Getty, 1981b; Yahner, 1978a). Home ranges may overlap significantly with one another, and encounters between

neighboring chipmunks are typically hostile, resulting in chasing or more rarely, physical fighting (Elliott, 1978; Yahner, 1978b). Typically, neighboring chipmunks will avoid one another (Getty, 1981b).

Breeding. Breeding takes place within two distinct seasons (Elliott, 1978; Pidduck & Falls, 1973; Smith & Smith, 1972): Immediately after emergence from hibernation in the spring, and again during mid-summer. The temporal delineation of breeding and non-breeding seasons may potentially differ among populations that geographically vary due to climatic differences (e.g. Adirondacks (Elliott, 1978) vs. Ontario (Pidduck & Falls, 1973; Smith & Smith, 1972)). Of relevance, a study of chipmunk breeding in a geographically nearby study area (Ottawa, ON area) found that the Spring Breeding season lasted from mid March to the end of April (Smith & Smith, 1972) and ceased during the month of May, when many females are carrying and delivering litters (Pidduck & Falls, 1973). This is followed by a second breeding season lasting from the beginning of June to the end of July. Generally, females will mate in one or the other breeding season, although they will occasionally mate twice in one year (Smith & Smith, 1972). Litters range in size from ~2-6 pups, which emerge from their mother's burrow at 5-7 weeks of age (Pidduck & Falls, 1973). During breeding seasons, males substantially increase their home range size (Bowers & Carr, 1992), making excursions into female territories in order to find mates (Elliott, 1978). Females, on the other hand, have similar, if not slightly smaller, home range sizes during breeding compared to non-breeding periods (Bowers & Carr, 1992).

Food-Caching. Chipmunks are primarily larder-hoarders, meaning that they bring foraged food back to their central burrow for storage (Elliott, 1978). Scatter-hoarding behaviour has also been observed in chipmunks, although to a lesser extent than larder-

hoarding (Clarke & Kramer, 1994; Elliott, 1978). Scatter-hoarding appears to occur more frequently in juveniles and females or when competition for food sources is introduced (Clarke & Kramer, 1994). Chipmunks typically forage for tree seeds, plant roots, and occasionally meat such as snails or even bird chicks. These sources are based on seasonal availability with no pronounced sex differences in food-caching behaviour (Elliott, 1978; Snyder, 1982).

Hibernation. Chipmunks enter torpor for several months during the winter (Maclean, 1981). The duration and depth of torpor depend on the size and nutritional content of their winter larders (Humphries, Kramer, & Thomas, 2003; Munro, Thomas, & Humphries, 2005). Individuals can occasionally be seen aboveground during short warm periods during winter. Spring emergence is signalled by increasing temperature (Elliott, 1978).

The current experiment. The present study aimed to further investigate sex and seasonal differences in the HPC of the Eastern Chipmunk, *Tamias Striatus*. Chipmunks were compared across three factors: Sex (male vs female), mating competency (breeding vs non-breeding), and activity phase (early active season (Apr-May) vs late active season (June-Oct)). Differences in home range size occur in chipmunks across sex and mating competency, meaning that differences in the HPC could be expected across these factors. In contrast, no differences in home range size occur between the early and late activity phase, meaning that no range size-driven changes in the HPC should have been observed across this factor. Thus activity phase served as a control for potential confounding variables such as the proximity to hibernation emergence. The study involved two main

components (chapter 2: hippocampal volumes; chapter 3: neurogenesis) with the following hypotheses:

1) Examining hippocampal volume. Male chipmunks have larger home ranges than females during the breeding periods with equivalent home range sizes during non-breeding periods (Bowers & Carr, 1992; Elliott, 1978). Therefore, it was predicted that hippocampal volume should be highest in males during the breeding condition. No sex difference was expected during the non-breeding condition, consistent with lack of sex or seasonal variation in home range size. Additionally, no differences in HPC volume were expected between the early and late activity phase, given that no changes in home range size occur between these two time periods.

2) Examining rates of hippocampal neurogenesis. Given that adult-born granule cells are needed for the formation of new spatial memories (Jessberger et al., 2009; Snyder et al., 2005), it was predicted that the rate of neurogenesis would be highest in males during the breeding condition, when males increase their range size during breeding (Bowers & Carr, 1992; Elliott, 1978). No sex difference was expected during the non-breeding condition, consistent with lack of sex or seasonal variation in home range size. Additionally, no differences in neurogenesis were expected between the early and late activity phase, given that no changes in home range size occur between these two time periods.

CHAPTER 2 - EXPERIMENT 1: SEX AND SEASONAL VARIATION IN HIPPOCAMPAL VOLUME

Introduction

Evidence from wild-living animals suggests that the volume of the HPC exhibits both sexual dimorphism and seasonal plasticity. Species, sex, and seasonal differences in the HPC volume of wild species correlate with spatial behaviours, including food-caching mating, and have been found in both passerine and corvid birds (Healy & Krebs, 1992; Krebs et al., 1989; Lucas, Brodin, de Kort, & Clayton, 2004; Sherry & Vaccarino, 1989; Smulders et al., 1995), cowbirds (Clayton et al., 1997; Reboresda et al., 1996; Sherry et al., 1993) various species of voles (Jacobs et al., 1990; Yaskin, 2013), shrews (Yaskin, 2005), and kangaroo rats (Jacobs & Spencer, 1994). Given that these differences correlate with sex and seasonal differences in space use related to food-caching and mating, among other factors, it is likely that variable requirements for spatial memory capacity result in concomitant variation in HPC volume (Jacobs, 1996; Sherry, 2006; Yaskin, 2011).

A number of species exhibit sex differences in space use specifically during seasonally-restricted breeding periods and presumably lead to intraspecific sex and seasonal differences in spatial memory requirements. In polygynous rodents, males tend to expand their home range size to maximize the potential to find mates (Behrends et al., 1986a, 1986b; Elliott, 1978; Gaulin & FitzGerald, 1988; Randall, 1991). In birds, female brood-parasitic cowbirds increase their space use by searching for target nests during breeding (Mason, 1987; Rothstein et al., 1987). However, the few studies that have analyzed both sex and seasonal differences in HPC volume within individual species have

not provided clear evidence that the HPC is always correlated both sexually and seasonally with space use.

Clayton et al. (1997) found that in brood-parasitic cowbirds, females had a larger HPC volume than males particularly during the breeding season, supporting the hypothesis that HPC volume and space use are correlated. Conversely, Burger et al. (2013) found that male Richardson's ground squirrels had a significantly larger HPC during the non-breeding season, which contradicts the general prediction that male HPC volume should be higher during the breeding season. In contrast to studies finding evidence of seasonal change, Lavenex et al. (2000) examined eastern gray squirrels and found that, despite there being a general sex difference in HPC volume favouring males, no seasonal change occurred. Overall, these studies do not appear to provide clear support for the hypothesis that HPC volume changes seasonally along with mating-related changes in space use.

A detailed examination of the particulars of each species' behavioural ecology and broader species differences may, however, provide insight into the differences noted above. For instance, Lavenex et al. (2000) postulate that previous findings of seasonal change in short-lived mammals reflect developmental processes rather than seasonal plasticity during adulthood, and that the HPC of long-lived mammals thus appears more static during adulthood. Burger et al. (2013) point out that, in the case of Richardson's ground squirrels, females tend to live in colonies during the breeding season, reducing the difficulty on the part of the males to find multiple mates and perhaps, by extension, decreasing the spatial aspects related to mating. Burger et al. (2013) also raise the possibility that the food caching behaviour that only male ground squirrels engage in during the fall, which is outside the breeding period, may be a more taxing spatial task than the requirements for

breeding. Further, ground squirrels hibernate and hibernation is associated with drastic changes in both dendritic morphology in the HPC (Popov et al., 2007), neurogenesis in HPC (Popov et al., 2011) and hippocampally-dependent behaviors (Weltzin et al., 2006). For instance, changes in context fear memory, a hippocampally-dependant behaviour, have been observed 24 hours after arousal from hibernation and return to normal within 4 weeks after arousal from hibernation (Weltzin et al., 2006). However, it is not known how long hippocampally-dependent memory functions remain altered after arousal from hibernation. Given that breeding in ground squirrels occurs immediately following arousal from hibernation effects of breeding on the HPC volume in this species is confounded with potential changes that occur in the HPC during hibernation. Thus, several confounds complicate the interpretation of changes in HPC volume and its relation to season, age, and breeding and non-breeding behaviors.

As discussed in an earlier section, the eastern chipmunk is an ideal species with which to address some of the confounds noted above that may have affected other studies, as well as to contribute data from a species that has not yet been investigated. The present experiment compared HPC volume across sex, mating competency, and activity phase in the eastern chipmunk. Given that chipmunks are a polygynous species, it was hypothesized that males would have a larger HPC volume than females, (Elliott, 1978; Smith & Smith, 1972). Further, given that males increase their range size during breeding seasons (Elliott, 1978; Smith & Smith, 1972), it was hypothesized that HPC volume of males would be larger during the breeding seasons than during the non-breeding seasons.

Methods

Animals

The research project and procedures were approved by the University of Ontario Institute of Technology Animal Care Committee, which adheres to the guidelines of the Canadian Council on Animal Care. A wildlife-trapping permit was also obtained from the Ontario Ministry of Natural Resources.

Fifty-one eastern chipmunks were collected over two consecutive years between March and November. The eastern chipmunk emerges from hibernation during March and breeds until the end of April. A second breeding season begins in June and extends to the end of July. From August until November, chipmunks hoard food in their burrows to prepare for winter (Elliott, 1978; Smith & Smith, 1972). Hence, based on this behavioural ecology, four distinct seasons were delineated: Early breeding (March-April), early non-breeding (May), late breeding (June-July), and late non-breeding (August-November). These seasons were grouped by mating competency (breeding vs non-breeding) as well as by whether they occurred early or late in the active season. The overall numbers of male and female chipmunks collected in each season are shown in Table 1.

Table 1.
Numbers of male and female chipmunks captured in each season.

Mating Competency:		Breeding		Non-breeding	
Relation to		Early	Late	Early	Late
Hibernation:					
Sex:	Male	9	7	9	10
	Female	4	7	2	3

The chipmunks were taken from various different collection sites in the vicinity of Peterborough, Ontario, Canada. A map of the collection sites is shown in Figure 1. Chipmunks were trapped using Havahart Live Chipmunk Traps (Lee Valley Tools, Canada). The traps were baited with either peanuts or sunflower seeds, placed at collection sites, and checked within 4 hours of having been set. Female chipmunks that showed obvious signs of pregnancy or lactation (e.g. large abdomen; large, red nipples) were released (n=2). Otherwise, chipmunks were transferred from the traps into a large wool sock so they could be restrained and euthanized.

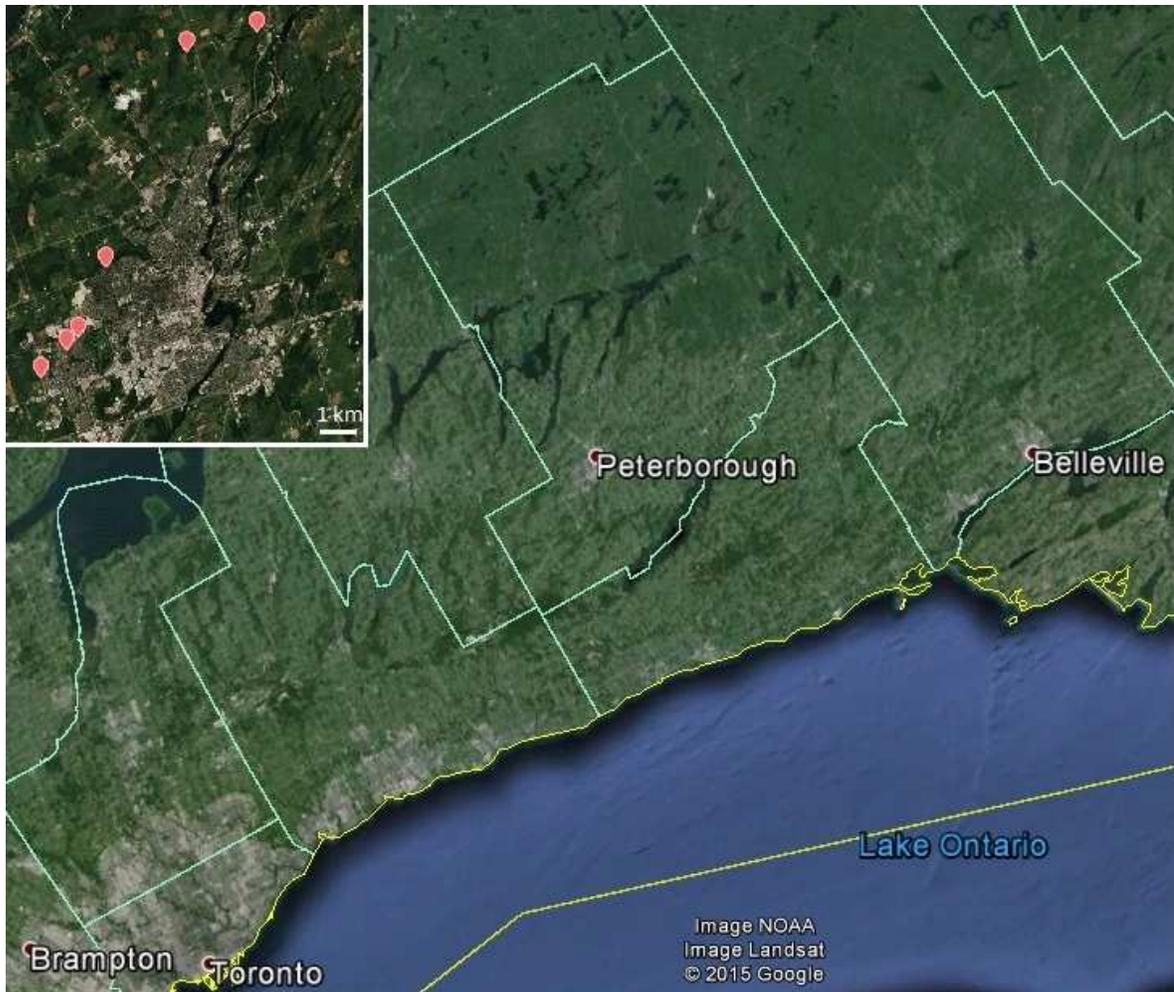


Figure 1. Map of Peterborough, ON showing the locations where chipmunks were caught.

Perfusions and Histology

Perfusions were conducted on site using a portable, gravity-driven perfusion system (AutoMate Scientific, Berkeley, CA). Chipmunks were removed from the traps and administered an intraperitoneal injection of sodium pentobarbitol (0.1 ml). After being weighed to the nearest 5 g with a Pesola hanging scale, chipmunks were then perfused intracardially with 100 mL of PBS followed by 50 mL of 4% paraformaldehyde. The brains were extracted and postfixed in 4% paraformaldehyde for 24 hours before being transferred to 30% sucrose/sodium azide for cryoprotection. After the brains were no

longer buoyant in the solution, they were sectioned on a freezing sliding microtome (American Optical Corporation, Buffalo, NY or; Leica Biosystems, Concord, Ontario, Canada) at 40 μm . The tissue was placed into tubes containing every 12th section through the whole brain (excluding the cerebellum). A series of sections through the HPC was mounted to gelatin-coated glass slides and stained with cresyl violet.

Age Estimation

Dentition. Chipmunks were categorized as adults, subadults, or juveniles by examining the upper molars according to the method described by (Smith & Smith, 1972). Specifically, adults can be distinguished from "subadults" by the presence of a permanent fourth upper premolar. In each chipmunk, the fourth upper premolar was examined to determine if it was deciduous (dP4) or permanent (pP4). The dP4 appears more triangular in shape, whereas the pP4 is more ovular in shape. The presence of a pP4 indicates that the animal is adult, whereas the presence of a dP4 or a pP4 that has only partially emerged indicates that the animal is subadult. Juveniles were characterized by the presence of the dP4 as well as the lack of a fully emerged third molar (M3).

Eye lens weight. The relative age of each chipmunk was also estimated using the dry weight of the eye lens. Eye lens weight is positively correlated with age and has been previously used to estimate age in several wild species (Augusteyn, 2014; Cavegn et al., 2013; Epp et al., 2009; Hardy et al., 1983). In the present study, it was used to provide a continuous relative age estimate. The lens of the left eye of each chipmunk was dissected out, dried for 3h in an oven at 75° C, and weighed to the nearest 0.1 mg. Eye lenses weighed and average of 13.1 mg (SD = 2.8).

Hippocampal Volume Estimation

All volumetric estimates were obtained using the Cavalieri point counting method (Mouton, 2002). Specifically, a grid of points was superimposed on each section, and grid points which contacted a given region of interest were counted. Volume was computed by multiplying the sum of all points counted by the section sampling fraction (1/12), the area per point, and the distance between sections. Estimates of the absolute volume of the HPC and the HPC volume relative to brain volume were obtained using the following quantification parameters:

Absolute HPC volume. A grid with an area per point of 0.2 mm^2 was superimposed at 2x magnification on every section at that contained the HPC. Any points that contacted HPC cell fields (CA1-3, DG) as well as HPC white matter (oriens layer, alveus) were counted.

Relative HPC Volume. In order to estimate the volume of HPC tissue relative to whole brain volume, a grid with an area per point of 3 mm^2 was superimposed at 1x magnification on each section that contained HPC tissue, and any point that contacted tissue anywhere within the section was counted. For each brain, a ratio of whole HPC volume to the volume of HPC-containing sections was computed.

Statistical Analysis

The data for absolute and relative HPC volume were analyzed using SPSS (V. 21; IBM Corporation, Armonk, NY). Data were compared across sex (male, female), mating competency (breeding, non-breeding) and activity phase (early, or apr-may; and late, june-oct). Thus the analysis was conducted with a 2x2x2 factorial design with the following independent variables: "sex", "mating competency", and "activity phase".

Results

Age and Body Measurements

The basic body measurements of chipmunks in each condition are shown in Table 2. Chipmunks weighed an average of 132.65 g (SD = 12.22) with body weights that were not significantly different between males (Mean = 131.14, SD = 12.90) and females (Mean = 135.94, SD = 10.20), $t(49) = -1.309$, $p = .197$. Analysis of dentition revealed that most chipmunks collected were adults. Four males and 2 females appeared to be subadults and 1 male appeared to be a juvenile. These animals were still included in the study given that body weight and lens weight could be used to control for age effects in the analyses. When age categorization from the dentition analysis was treated as an ordinal variable, significant Spearman correlations were found between dentition and lens weight ($r_s = .58$, $p < .05$) and body weight ($r_s = .57$, $p < .05$). Additionally, lens weight and body weight were positively correlated (Pearson $r = .67$, $p < .05$). The intercorrelations between dentition, body weight, and lens weight suggested that both body weight and lens weight could be used as continuous age variables, but lens weight was preferred as the primary age variable upon which to base conclusions given that it would not be affected by food abundance, whereas body weight can vary independent of age due to differences in food intake.

Sex and Seasonal Analysis of Body Measurements

Body weight. A 2x2x2 ANOVA conducted on body weight with sex (male, female), activity phase (early, late), and mating competency (breeding, non-breeding) as between subjects variables failed to reveal significant main effects, all F values < 2.245 , p values $> .14$, or any interactions, all F values < 1.791 , p values $> .187$.

Lens weight. A 2x2x2 ANOVA conducted on lens weight using the same between-subjects variables found a significant main effect of activity phase, $F_{(1,43)} = 4.183$, $p = .047$, with lens weights being higher in the early activity phase ($M = 14.235$ mg, $SE = .609$) than the late activity phase ($M = 12.593$, $SE = .524$), suggesting that chipmunks caught during the late activity phase may have been younger. No main effect of sex ($F_{(1,43)} = 2.834$, $p = .1$) or mating competency ($F_{(1,43)} = .099$, $p = .754$) was found on lens weight, nor were there any significant interactions, all F values < 3.176 , p values $> .06$.

Brain weight. A 2x2x2 ANOVA conducted on brain weight revealed no main effects of sex, $F_{(1,43)} = 0.131$, $p = .72$, or activity phase, $F_{(1,43)} = 0.916$, $p = .344$. However, a main effect of mating competency was found, $F_{(1,43)} = 5.83$, $p = .02$, whereby brain weight was greater in the non-breeding condition than in the breeding condition. No interactions were significant, all F values < 1.87 , all p values $> .178$.

Table 2.
Mean Body, Brain, and Eye Lens Weights for Each Group

Mating Competency:		Breeding		Non-breeding		
		Early	Late	Early	Late	
Relation to						
Hibernation:						
Sex:	Male	Body Weight (g)	M = 133.33 SD = 15.21	M = 136.67 SD = 10.0	M = 122.14 SD = 17.53	M = 130.5 SD = 5.5
		Brain Weight (g)	M = 2.21 SD = 0.15	M = 2.19 SD = 0.13	M = 2.30 SD = 0.12	M = 2.45 SD = 0.15
		Lens Weight (mg)	M = 14.47 SD = 2.46	M = 14.2 SD = 1.95	M = 11.99 SD = 4.14	M = 10.3 SD = 0.35

Female	Body Weight (g)	M = 131.25	M = 140.0	M = 136.43	M = 138.33
		SD = 6.29	SD = 0.00	SD = 13.45	SD = 10.41
	Brain Weight (g)	M = 2.25	M = 2.35	M = 2.21	M = 2.43
		SD = 0.11	SD = 0.12	SD = 0.15	SD = 0.12
	Lens Weight (mg)	M = 14.63	M = 13.65	M = 13.09	M = 15.0
		SD = 2.73	SD = 0.07	SD = 3.05	SD = 0.95

Absolute HPC Volume

Representative images of the chipmunk brain and HPC are shown in Figure 2. A 2x2x2 ANOVA with sex, activity phase, and mating competency as between-subjects variables was performed on the absolute volume of the HPC (Figure 14, Appendix). ANOVA revealed a main effect of mating competency, $F_{(1,43)} = 4.097, p = .049$, with larger absolute hippocampal volumes during the non-breeding period ($M = 12.63 \text{ mm}^3, SD = 0.93$) than during the breeding period ($M = 11.98 \text{ mm}^3, SD = 0.73$). Interestingly, neither the main effect of sex ($F_{(1,43)} = 2.02, p = .16$) nor activity phase ($F_{(3,43)} = 1.82, p = .18$) reached significance. No interactions reached significance either, all F values < 0.241 , all p values $> .62$.

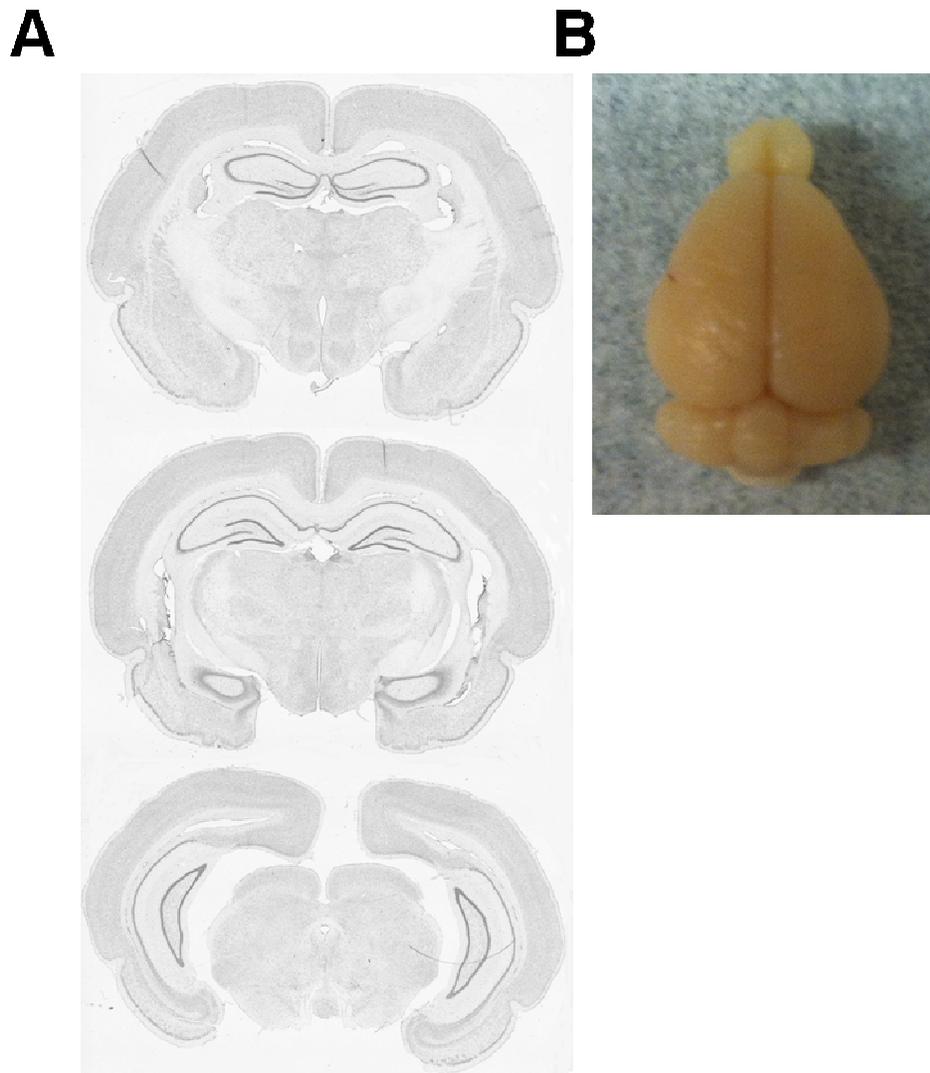


Figure 2. A) Representative sections showing the chipmunk HPC from anterior (top) to posterior (bottom). B) A representative chipmunk brain (anterior at top, posterior at bottom).

Controlling for Body Weight

A 2x2x2 ANCOVA using *the same between subjects variables as above and* body weight as a significant covariate ($F_{(1,42)} = 11.984, p < .001$; ANCOVA-adjusted means are displayed in Figure 3) revealed a main effect of sex, $F_{(1,42)} = 5.403, p = .025$, with males

having larger HPC volumes (adjusted $M = 12.498 \text{ mm}^3$, adjusted $SE = .132$) than females (adjusted $M = 11.905 \text{ mm}^3$, adjusted $SE = 0.215$). However, controlling for body weight reduced the effect of mating competency reported above to non-significant, $F_{(1,42)} = 2.181$, $p = .147$. As well, there was no main effect of activity phase, $F_{(1,42)} = 1.068$, $p = .31$, nor were there any interactions observed among the variables, all F values < 0.441 , all p values $> .509$.

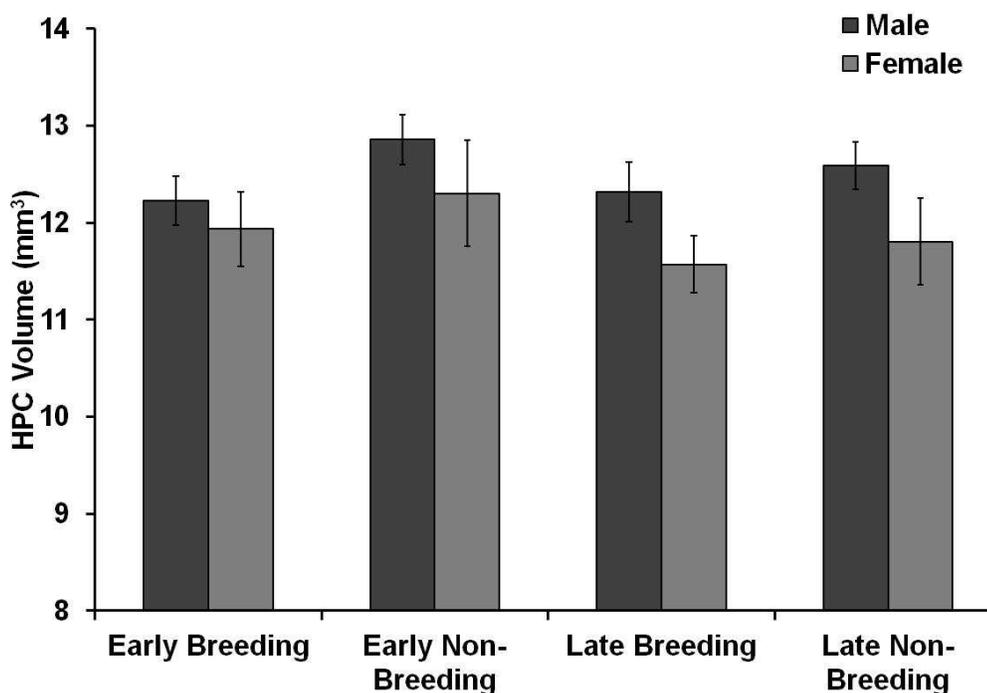


Figure 3. Mean (\pm SEM) absolute volumes of the HPC in male and female chipmunks. Displayed means are corrected for body weight. Males had a larger HPC than females, with no effects of mating competency or activity phase.

Controlling for Lens Weight

A 2x2x2 ANCOVA using the same between subjects variables as above and lens weight as a significant covariate ($F_{(1,42)} = 7.998, p = .007$; ANCOVA-adjusted means are displayed in Figure 4) revealed a main effect of sex, $F_{(1,42)} = 4.79, p = .034$, with males having larger HPC volumes (adjusted $M = 12.479$, adjusted $SE = .137$) than females (adjusted $M = 11.895 \text{ mm}^3$, adjusted $SE = 0.226$) and a main effect of mating competency, $F_{(1,42)} = 5.363, p = .026$, with non-breeding chipmunks having larger HPC volumes (adjusted $M = 12.487 \text{ mm}^3$, adjusted $SE = .203$) than breeding chipmunks (adjusted $M = 11.895 \text{ mm}^3$; adjusted $SE = 0.226$). As above, there was no main effect of activity phase, $F_{(1,42)} = 0.299, p = .588$, nor were there any interactions observed among the variables, all F values < 0.597 , all p values $> .444$.

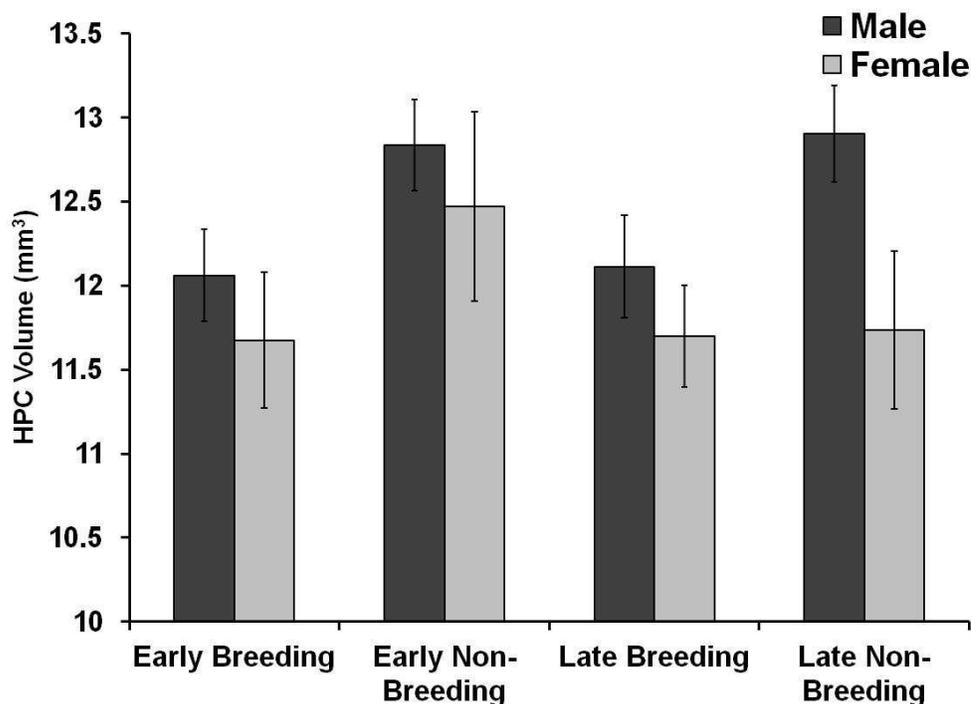


Figure 4. Mean (\pm SEM) absolute volumes of the HPC in male and female chipmunks.

Displayed means are corrected for lens weight. Males had a larger HPC than females, and

non-breeding chipmunks of both sexes had a larger HPC than breeding chipmunks. HPC volume did not differ significantly between the early and late activity phase.

Relative HPC volume

A 2x2x2 ANOVA with sex (male, female), activity phase (early, late), and mating competency (breeding, non-breeding) as between-subjects variables was performed on the ratio of the volume of the HPC relative to brain volume (Figure 15, Appendix). ANOVA failed to reveal any main effects of sex, season, or mating competency, all F values < 0.843, all p values > .363, or any interactions significant, all F values < 0.23, all p values > .64.

Controlling for Body Weight

A 2x2x2 ANCOVA using the same between-subjects variables as above and body weight as a significant covariate ($F_{(1,42)} = 13.751, p = .001$; ANCOVA-adjusted means are displayed in Figure 5) failed to reveal a main effect of sex, activity phase, or mating competency, F values < 3.403, p values > .074. Additionally, no interactions reached significance, all F values < 0.328, p values > .367.

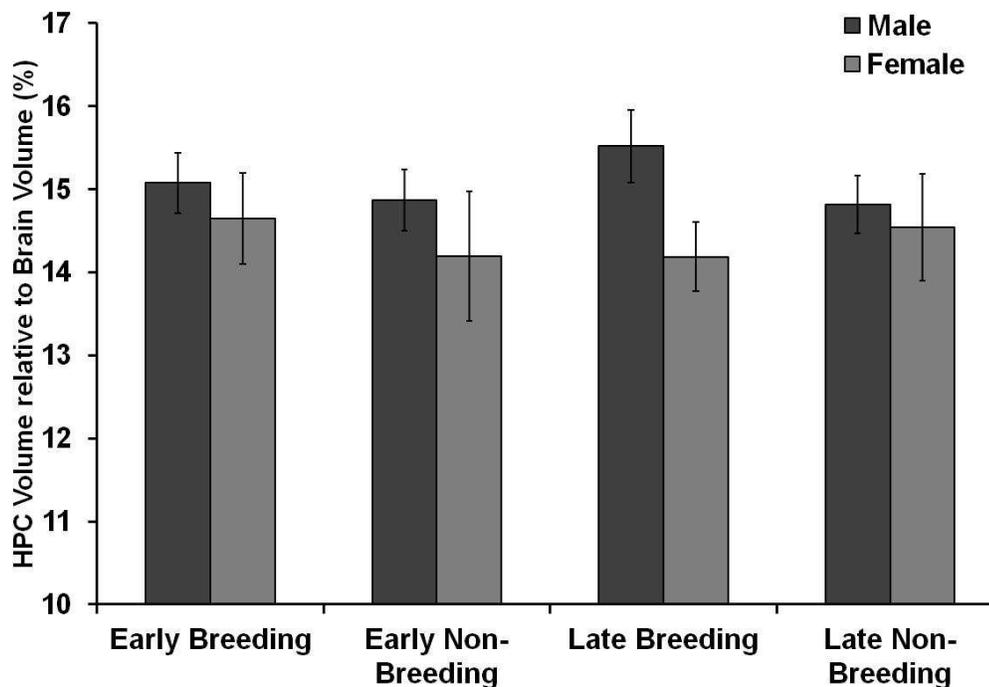


Figure 5. Mean (\pm SEM) proportions (%) of the HPC to surrounding tissue in males and females. Displayed means are corrected for body weight. No effects of sex, mating competency, or activity phase were found.

Controlling for Lens Weight

A 2x2x2 ANCOVA using the same between-subjects variables as above and lens weight as a significant covariate ($F_{(1,42)} = 21.481, p < .001$; ANCOVA-adjusted means are displayed in Figure 6) revealed a main effect of sex, $F_{(1,42)} = 4.983, p = .031$, with males having a larger proportion of HPC tissue (adjusted M = 15.069%, adjusted SE = .176) than females (adjusted M = 14.299%, adjusted SE = 0.291). The analysis failed to find main effects of mating competency, $F_{(1,42)} = .107, p = .746$, or activity phase, $F_{(1,42)} = 1.134, p = .293$, and no interactions were observed among the variables, all F values < 0.815 , all p values $> .371$.

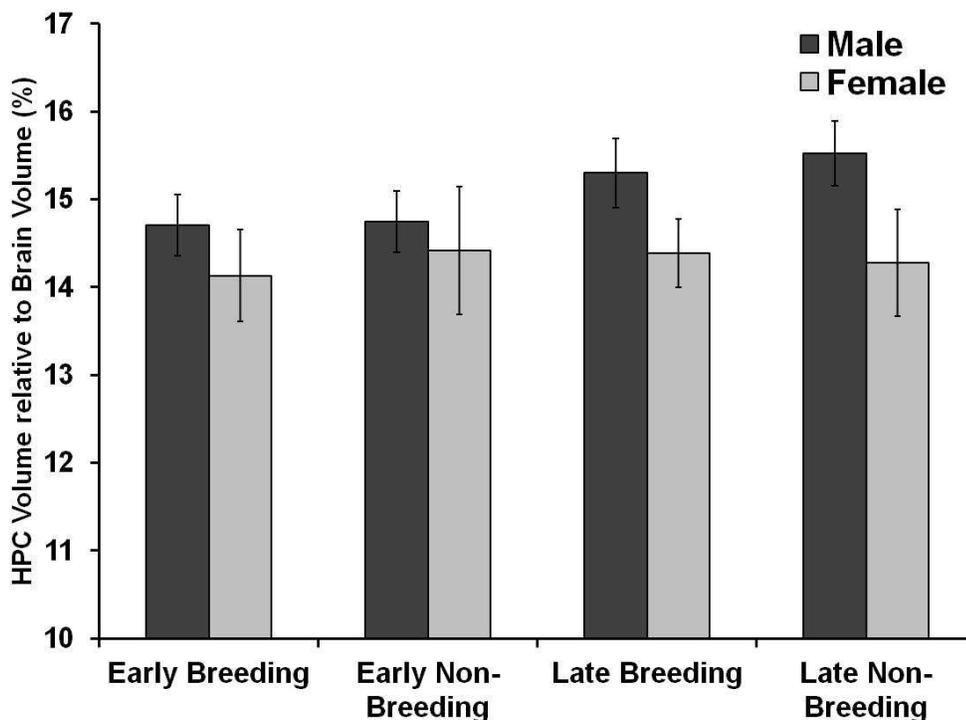


Figure 6. Mean (\pm SEM) proportions (%) of the HPC to surrounding tissue in males and females. Displayed means are corrected for lens weight, and reveal that males had a larger proportion of HPC tissue to total brain volume than females after controlling for age, with no effects of mating competency or activity phase.

Discussion

The present experiment investigated sex and seasonal differences in the volume of the HPC in the eastern chipmunk, *Tamias Striatus*. It was predicted that HPC volume would be larger in males than in females specifically during breeding periods as male chipmunks increase the size of their home range during breeding (Bowers & Carr, 1992; Elliott, 1978), ostensibly increasing their requirement for spatial memory capacity. This hypothesis was partially confirmed by the finding that, after controlling for age differences and total brain volume, relative HPC volume was larger in males than in females. The

observed overall sex difference in HPC volume comports with previous studies finding a sex difference in HPC volume favouring males of polygynous rodents during the breeding season (Jacobs et al., 1990; Jacobs and Spencer, 1994; Lavenex et al., 2000).

However, fluctuation between breeding and non-breeding conditions was only seen in absolute HPC volume, an effect potentially related to the present finding that total brain weight exhibited fluctuation between the breeding and non-breeding conditions. After controlling for total brain volume, relative HPC volume remained stable across breeding conditions and activity phase. Thus, the fluctuation in absolute HPC volume was likely related to volume changes across the whole brain. Though this finding may be of significance, the most accurate approach to the current question was to examine differences in the proportion of brain tissue devoted to the HPC, given that absolute HPC volume could vary purely by virtue of differences in total brain volume, rather than differences in the amount of metabolic resources allocated to support spatial memory function *per se*. Furthermore, absolute HPC volume was larger in the non-breeding season rather than the breeding season, and this effect was observed in both sexes, inconsistent with the hypothesis that males would have a larger HPC volume during the breeding season. This result is somewhat similar to the finding of Burger et al. (2013) that HPC volume in Richardson's ground squirrels was largest in non-breeding males. However, Burger et al. (2013) were considering HPC volume relative to total brain volume, whereas the present analysis found no evidence of fluctuation in relative HPC volume. Therefore, though the present results are consistent with the prediction that males would have a larger HPC, they provide no support for the idea that HPC volume is seasonally-plastic and grows in to support increased range size during mating.

The present lack of seasonal change in the relative HPC volume of eastern chipmunks contradicts the general hypothesis that male HPC volume increases during breeding in polygynous rodents (Burger et al., 2013; Jacobs et al., 1990; Jacobs, 1996). A potential explanation for the lack of seasonal differences offered by Lavenex et al. (2000) may also apply to the present results. Lavenex et al. (2000) postulated that the seasonal differences observed in several species are manifestations of developmental processes, and that in longer-lived mammals such as the eastern gray squirrels in their study, these seasonal differences subside during adulthood. Similarly, we examined a rodent with a relatively long lifespan. Eastern chipmunks can live 2-3 years in the wild (Tryon & Snyder, 1973). Although the absolute age of the chipmunks in the present study could not be determined, most appeared to be adults based on analysis of the upper molars. However, chipmunks exhibit fully adult molars by 3 months of age (Smith & Smith, 1972), meaning that absolute age classification of wild specimens beyond 3 months old cannot be determined from this method. Nonetheless, it is likely that many of the chipmunks in the current sample were at least a year old, especially those captured during the spring, which would have includes those that had overwintered and therefore born the year before or earlier. If the majority of the present chipmunk sample contained fully mature adults, the lack of seasonal change in relative HPC volume could corroborate the view that HPC volume is seasonally stable in mature individuals of long-lived species.

An additional possibility is that in chipmunks, spatial memory capacity requirement remains relatively constant during the animals' active period. Given that chipmunks engage in a spring and summer breeding season separated by roughly one month (Smith & Smith, 1972), there may be no adaptive reason for the HPC to fluctuate significantly in volume

from the spring emergence to end of summer. Thus, the male chipmunk HPC may remain in 'breeding condition' for the entire summer. Indeed, every single adult male captured before the end of July had scrotal testes, including during the early non-breeding season in May, which lends a small amount of support for this idea in that males appear to remain in physical breeding condition even during May.

Moreover, chipmunks forage for food and engage in both larder- and scatter-hoarding throughout their active period (Elliott, 1978), and previous research has implied that in some species, the spatial demands of food-caching can have a greater influence on hippocampal morphology than home range size increases during mating (Burger et al., 2013). Chipmunks occasionally make long excursions to food sources outside their normal home range, including during non-breeding periods (Elliott, 1978). Additionally, chipmunks have been found to engage in brief periods of intensified food-caching during the fall (Humphries, Thomas, Hall, Speakman, & Kramer, 2002). These bouts of intense food-caching do not last for the entire fall, but it is perhaps possible that the HPC maintains its volume in order to accommodate this behaviour. Therefore, it is possible that seasonal differences in spatial behaviour during mating do not exert an influence on the HPC that is significantly above and beyond other spatial demands such as foraging behaviour.

Importantly, the present experiment calculated relative HPC volume as a percentage of the coronal slab brain tissue beginning and ending at the anterior and posterior boundaries of the HPC. Therefore, parts of the whole brain were excluded from this normalization of HPC volume under the assumption that brain volume would vary between individuals more or less evenly across brain areas. One possibility is that the entire chipmunk brain, including the HPC, fluctuates across seasons, a possibility that is

corroborated by the present finding that chipmunk brain weight in both sexes was greatest during the non-breeding condition. Indeed, chipmunk cranial capacity increases with habitat complexity (Budeau & Verts, 1986). However, this slab of tissue also contains large portions of the perirhinal and entorhinal cortices, which have strong connections to the HPC and play important roles in memory and spatial navigation, including the role of the perirhinal cortex in object recognition and the presence of spatial grid cells in the entorhinal cortex (Aggleton & Brown, 2005; Moser et al., 2008; Squire & Zola-Morgan, 1991). Thus, an interesting possibility is that these areas also undergo plastic changes across seasons to compensate for seasonal differences in memory demands. Recently, it was found that the volume of the entorhinal cortex varies by season in Richardson's ground squirrel, along with several other regions (Keeley, Burger, Saucier, & Iwaniuk, 2015), lending credence to this hypothesis. If the HPC and surrounding memory-related cortices concurrently fluctuate in volume in the same direction, it would be consistent with the attenuation of seasonal HPC volume changes when their volumes are used as a control variable. However, it remains unclear what would cause multiple spatial memory-related brain areas to increase in volume during the period opposite the increase in range size exhibited by males.

The present results support the idea that sex differences in space use related to mating systems are associated with corresponding differences in HPC volume. However, no evidence of seasonal change in HPC volume was observed after controlling for age and normalizing HPC volume to account for differences in brain volume. This lack of seasonal change may be the result of insufficient seasonal variation in spatial memory requirements during the active period, either because of an extended mating period involving two

closely-spaced breeding seasons, or spatially-demanding food-foraging outside the breeding seasons. It may also be the case that, as with Lavenex et al. (2000), the present results are evidence that seasonal fluctuations in HPC volume tend not to occur in long-lived animals. Alternatively, the memory-related cortices surround the HPC may also change in volume.

CHAPTER 3 - EXPERIMENT 2: SEX AND SEASONAL VARIATION IN HIPPOCAMPAL NEUROGENESIS

Introduction

Adult hippocampal neurogenesis has been observed across a variety of species (Barker et al., 2011). Although its exact functional role is not fully understood, there is strong evidence that neurogenesis is important in the formation and maintenance of hippocampal-dependent long-term memory (Marín-Burgin & Schinder, 2012). Neurogenesis is increased by spatial learning in the laboratory (Ambrogini et al., 2000; Epp et al., 2010; Gould et al., 1999), and can thus be predicted to increase with the performance of cognitively-demanding spatial behaviour in a naturalistic environment as well. Accordingly, several studies of wild-living animals have indicated species and seasonal differences in neurogenesis correlating to food-caching behaviour (Barker et al., 2005; Barnea & Nottebohm, 1994; Hoshooley & Sherry, 2007; Pan et al., 2013) as well as climactic harshness (Chancellor, Roth, LaDage, & Pravosudov, 2011) and elevation (Freas, LaDage, Roth, & Pravosudov, 2012). Both climactic harshness and elevation lead to greater spatial demands for food-caching. However, there are also studies that fail to find a

relationship between neurogenesis and food-caching (Hoshooley & Sherry, 2004; Johnson et al., 2010).

Although evidence of a lack of correlation between neurogenesis and food-caching in some studies would, on the surface, seem to contradict the idea that rates of neurogenesis fluctuate in response to changing cognitive demands, several other factors influencing rates of neurogenesis are present in wild-living populations aside from space use during food-caching. Rates of hippocampal neurogenesis are: increased by physical activity (van Praag et al., 1999) and sexual experience (Leuner et al., 2010); reduced by stress (Brummelte & Galea, 2010; Gould et al., 1997; Wong & Herbert, 2006) and aging (Amrein et al., 2011, 2004; Barker et al., 2005; Epp et al., 2009; Kuhn et al., 1996); and are affected differentially by gonadal hormones (Galea, 2008). Additionally, food-caching is not the only cognitively-demanding spatial behaviour that wild-living species engage in. Neurogenesis may also be influenced by changes in spatial cognitive demand due to the increase in male home range size during the breeding season in polygynous rodents (Behrends et al., 1986a, 1986b; Elliott, 1978; Gaulin & FitzGerald, 1988; Randall, 1991). Specifically, increased range size should lead to increased neurogenesis to support greater spatial memory capacity, leading to both sex and seasonal differences in neurogenesis in wild-living polygynous rodents.

As discussed in Chapter 1, only three studies have investigated sex and seasonal variation in neurogenesis in wild-living rodents, and each of these studies has produced different results. Galea and McEwen (1999) found that neurogenesis was higher in non-breeding female meadow voles than in males or breeding females. Burger et al. (2014) found that, like in Galea and McEwen (1999), neurogenesis was higher in non-breeding

Richardson's ground squirrels than in their breeding conspecifics, but it was the males that had higher neurogenesis than females. This difference was observed during the non-breeding season for ground squirrels, a season wherein Galea and McEwen (1999) found no sex difference. Contrary to both these studies, Lavenex et al. (2000) found no sex or seasonal differences in the eastern gray squirrel. Not only do these studies contradict one another, they find no evidence that neurogenesis increases during the breeding periods when the requirement for spatial memory is greater.

There are important differences between these studies that may explain the discrepant findings. As previously discussed in Chapter 2, male Richardson's ground squirrels engage in much greater space use than females even outside of the breeding season, which might obscure sex and seasonal differences related specifically to reproductive behaviour (Burger et al., 2013). There are also significant differences in the average life spans of the species that have been studied, with eastern gray squirrels living significantly longer in the wild, a potential confound for reasons also discussed in Chapter 2 (Lavenex et al., 2000b). Additionally, Galea and McEwen (1999) and Lavenex et al. (2000) assayed neurogenesis by injecting captured animals with H^3 -Thymidine or Bromodeoxyuridine (BrdU) and housed them in a laboratory setting for 48 hours before sacrificing them, whereas Burger et al. (2014) labelled immature neurons for doublecortin (DCX) after perfusing them in the field immediately following capture. Captivity can cause stress-induced changes in neurogenesis in wild-caught animals (Chawana et al., 2014), which may then have affected the findings of Galea and McEwen (1999) and Lavenex et al. (2000), but not Burger et al. (2014). Furthermore, the different methodologies for detecting newly born cells could produce differences in the age of the labelled neurons due to the fact

that exogenous markers such as H^3 -Thymidine would only label neurons that were born after the animals were captured and injected, whereas DCX labelling targets immature neurons born days or weeks before the animal is sacrificed (Brown et al., 2003). Therefore, multiple methodological and species differences could account for the disagreement in the findings of these three studies.

The examination of more species may help to clarify the relationship between range size and neurogenesis in wild-living populations. In particular, the eastern chipmunk is an ideal species in which to conduct such an analysis. As has been previously described, eastern chipmunks are polygynous and engage in two breeding seasons in which males increase their space use (Elliott, 1978; Smith & Smith, 1972). Additionally, both sexes exhibit similar home range sizes during the non-breeding seasons, and display similar food-caching behaviour (Elliott, 1978). The present experiment investigated sex and seasonal differences in hippocampal neurogenesis in the eastern chipmunk. It was hypothesized that neurogenesis would increase in males during the breeding season to accommodate increased spatial cognitive demand, but remain constant in females. Additionally, given that chipmunks exhibit little or no sexual dimorphism in space use during non-breeding periods (Bowers & Carr, 1992; Elliott, 1978), it was hypothesized that there would be no sexual dimorphism in neurogenesis during the non-breeding periods. There are also no marked differences in home range size between the early and late activity phase. Thus, neurogenesis should be equivalent across these two periods.

Methods

Animal collection, perfusions, histology, and body measurements were performed as described in Experiment 1.

Immunohistochemistry

A series of sections through the hippocampus was used to conduct fluorescence immunohistochemistry. The tissue was labelled for the protein doublecortin (DCX). The tissue was rinsed in 0.1% sodium azide for 8-10 minutes before undergoing a 48-h incubation in a 1:200 primary goat anti-DCX antibody (Santa Cruz Biotechnology, Inc., Dallas, TX) and Triton-X solution at room temperature. The tissue was then rinsed three times for 8-10 min in phosphate buffered saline (PBS) and transferred to a 1:1000 secondary mouse anti-goat antibody (Cy3 (red); Jackson Immuno Research Labs, West Grove, PA) and incubated for 24 h. Sections were then mounted in PBS onto glass slides and coverslipped immediately using Invitrogen Slow Fade™ Gold mounting medium with DAPI (Life Technologies, Burlington, ON).

Stereology

DCX cell counts. The estimate for DCX-positive cells for each brain was obtained according to unbiased/assumption-free stereology practices using the disector principle (Sterio, 1984). A grid of dissectors with a spacing of 150 μm was superimposed on images of each section containing the dentate gyrus (8-12 sections per brain) at 4x magnification. At each disector that contacted the dentate gyrus, magnification was increased to 100x, and DCX-positive cells were counted within a 5700 μm^2 optical fractionator. Only cells within the middle 15 μm of the tissue were counted despite the section thickness averaging 36.32 μm (SD = 2.10) at the time of quantification. This provided a guard height greater than 5 μm in all sections to avoid quantifying near the cut surfaces of the sections where cells may be cleaved/removed by the blade of the microtome. Additionally, only the tops of cells that came into focus within the middle 15 μm of tissue were counted. These parameters were

used to assure that at least 200 DCX-positive cells were counted in the dentate gyrus, which has been shown to be an ideal number of counted objects to obtain accurate and reliable estimates within a reference space (Mouton, 2002). Finally, the number of cells counted was multiplied by the inverse of 1) the respective section sampling fraction, 2) the area sampling fraction, and 3) the thickness sampling fraction to obtain the estimate of the total number of DCX-positive cells in the dentate gyrus.

Granule cell counts. Estimates of the total number of granule cells throughout the DG were obtained in order to normalize the DCX-positive cell counts to total cell granule cell counts. At 4x magnification, a grid of dissectors with a spacing of 200 μm was superimposed on each section containing the DG. At each disector contacting the DG, granule cells were counted within a 1250 μm^2 optical fractionator.

Dentate gyrus volume. Dentate gyrus volume was obtained using the Cavalieri point counting method as described in Chapter 2. A grid with an area per point of 0.0225 mm^2 was superimposed at 4x magnification on every section that contained the DG. Any points that contacted the granular layer of the DG were counted.

Statistical Analysis

Data were analyzed in the same manner as in Experiment 1.

Results

Absolute Estimates of DCX+ Cells

Sex and Seasonal Analysis. Extensive DCX labelling was achieved and produced cell count estimates ranging from ~1000 - ~90,000 DCX-positive cells in the DG (Figure 7). A 2x2x2 ANOVA with sex (male, female), mating competency (breeding, non-

breeding), and activity phase (early, late) as between-subjects variables was performed on the mean absolute estimates of DCX-positive cells (Figure 16, Appendix) and failed to find main effects of sex, $F_{(1,43)} = 1.387, p = .245$, activity phase, $F_{(1,43)} = 1.206, p = .278$, or mating competency, $F_{(1,43)} = .883, p = .353$. A significant sex*activity phase interaction was found, $F_{(1,43)} = 6.944, p = .012$, with males in the late activity phase having more DCX-positive cells than any other group ($M = 47.858 \times 10^3$ cells, $SE = 4.584 \times 10^3$). No other interactions reached significance, all F values < 1.902 , p values $> .174$.

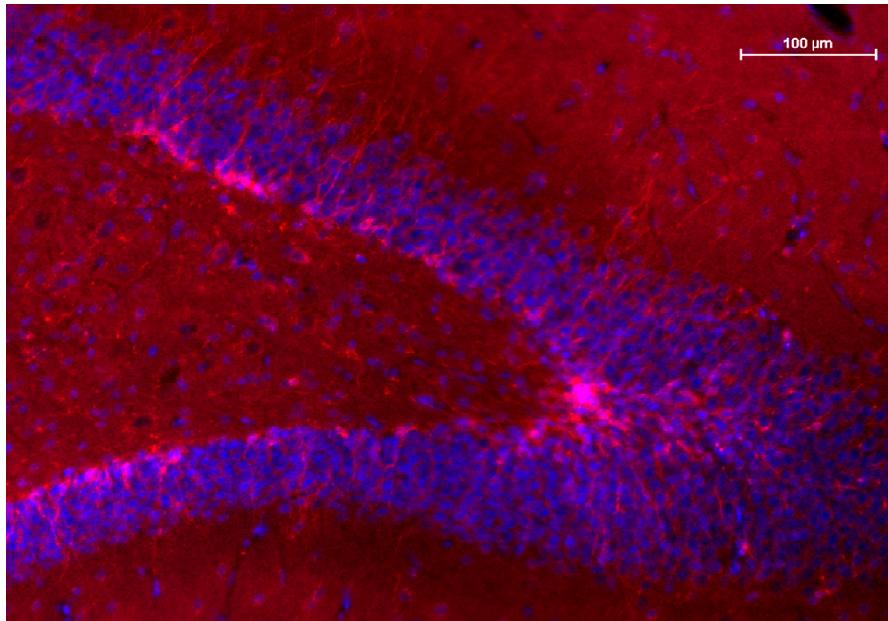


Figure 7. Photomicrograph of the chipmunk DG showing DCX-positive cells (red) and counterstained with DAPI (blue).

Controlling for Body Weight

A significant negative correlation was found between DCX-positive cell estimates and body weight, $r_{(51)} = -.60, p < .001$ (ANCOVA-adjusted means are displayed in Figure 8). A 2x2x2 ANCOVA using the same subject variables as above and body weight as a significant covariate ($F_{(1,42)} = 26.533, p < .001$) failed to find a main effect of sex, $F_{(1,42)} =$

.091, $p = .764$, or activity phase, $F_{(1,42)} = .466$, $p = .499$. However, a significant main effect of mating competency was found, $F_{(1,42)} = 5.101$, $p = .029$, where non-breeding chipmunks (adjusted $M = 39.065 \times 10^3$, $SE = 4.003$) had greater neurogenesis than breeding chipmunks (adjusted $M = 27.486 \times 10^3$ cells, $SE = 3.119$). Additionally, the sex*activity phase interaction remained significant from the non-age-controlled analysis, $F_{(1,42)} = 4.972$, $p = .031$, where neurogenesis was highest in males during the late activity phase (adjusted $M = 41.476 \times 10^3$ cells, $SE = 3.993$), $LSD p = .01$. No other significant interactions were found, all F values < 3.474 , p values $> .68$.

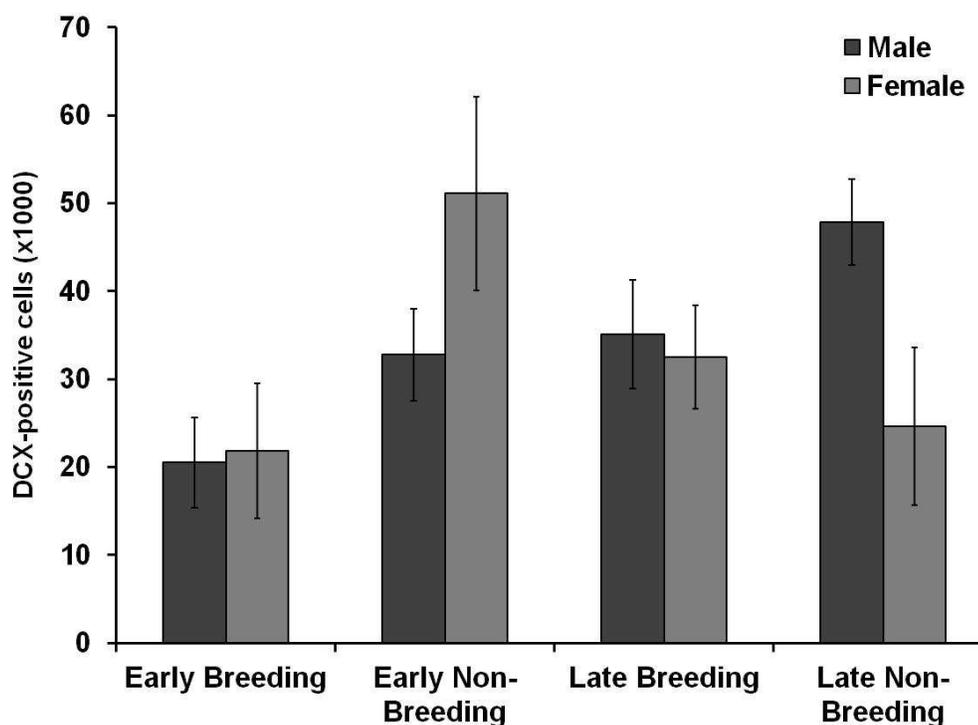


Figure 8. Mean estimates of DCX-positive cells (\pm SEM). Displayed means are corrected for body weight. No effect of sex was found, but neurogenesis was found to increase in males from the early to the late activity phase. Additionally, neurogenesis was higher during non-breeding than during breeding.

Controlling for Lens Weight

Absolute estimates of DCX-positive cells were strongly negatively correlated with lens weight, $r_{(51)} = -.817$, $p < .001$ (ANCOVA-adjusted means are displayed in Figure 9). A 2x2x2 ANCOVA using the same subject variables as above and lens weight as a significant covariate ($F_{(1,42)} = 60.033$, $p < .001$) attenuated all previous effects, failing to find main effects of sex, activity phase, or mating competency, all F values < 1.154 , p values $> .288$ or any significant interactions, all F values < 2.923 , p values $> .94$.

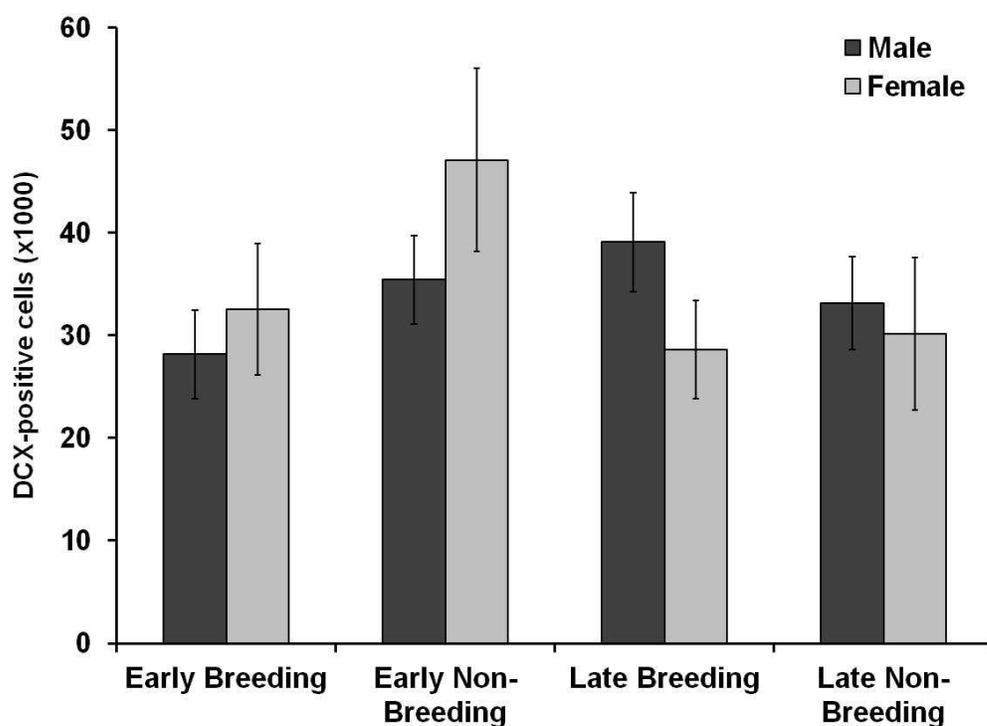


Figure 9. Mean estimates of DCX-positive cells (\pm SEM). Displayed means are corrected for lens weight. No effects of sex, mating competency, or activity phase were found.

Estimates of DCX+ Cells Relative to Total Granule Cells

Ratios of the number of DCX+ cells to the total number of granule cells in the DG, hitherto referred to as "relative neurogenesis", were compared in a 2x2x2 ANOVA (Figure

17, Appendix) and failed to find main effects of sex, activity phase, or mating competency, all F values < 2.503 , p values $> .12$. However, a significant sex*activity phase interaction was found, $F_{(1,42)} = 4.721$, $p = .035$, where males had higher relative neurogenesis in the late activity phase ($M = 1.992\%$, $SE = .213$) than in the early activity phase ($M = .935\%$, $SE = .204$), $LSD p = .001$. No other interactions reached significance, all F values < 3.004 , p values $> .069$.

Controlling for Body Weight

Relative estimates of neurogenesis were significantly negatively correlated with body weight, $r_{(51)} = -.63$, $p < .001$ (ANCOVA-adjusted means displayed in Figure 10). A 2x2x2 ANCOVA using the same subject variables as above and body weight as a significant covariate ($F_{(1,42)} = 35.183$, $p < .001$) revealed a main effect of mating competency, $F_{(1,42)} = 4.154$, $p = .049$, where relative neurogenesis was higher in the non-breeding (adjusted $M = 1.618\%$, $SE = .168$) than in the breeding condition (adjusted $M = 1.179\%$, $SE = .131$). The analysis failed to find a main effect of sex, $F_{(1,42)} = 0.09$, $p = .766$, or activity phase, $F_{(1,42)} = 1.702$, $p = .199$. A significant activity phase*mating competency interaction was found, $F_{(1,42)} = 5.889$, $p = .02$, where relative neurogenesis was lower in the early breeding condition (adjusted $M = .786\%$, $SE = .194$) than the early non-breeding condition (adjusted $M = 1.734\%$, $SE = .257$), $LSD p = .005$. No other interactions reached significance, all F values < 3.919 , p values $> .053$.

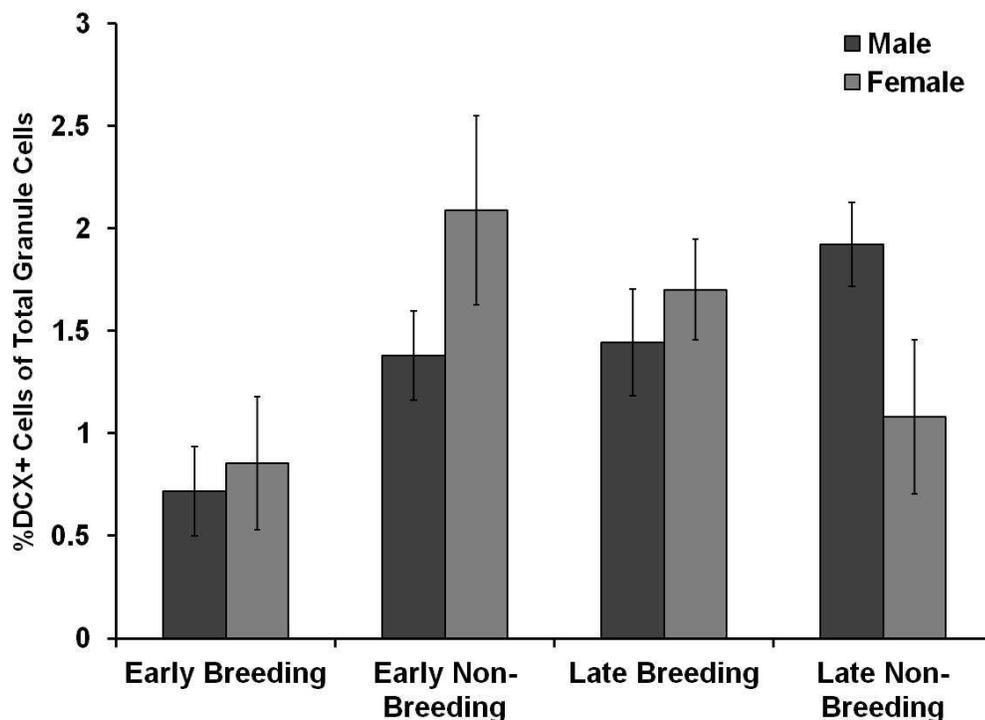


Figure 10. Mean (\pm SEM) numbers of DCX-positive cells relative to the total number of granule cells. Displayed means are corrected for body weight. No sex difference was found, but relative neurogenesis was lower in the breeding than in the non-breeding condition, particularly during the early activity phase, where this difference was most pronounced.

Controlling for Lens Weight

Relative neurogenesis estimates were strongly negatively correlated with lens weight, $r_{(51)} = -.803$, $p < .001$ (ANCOVA-adjusted means are displayed in Figure 11). A 2x2x2 ANCOVA using the same subject variables as above and lens weight as a significant covariate ($F_{(1,42)} = 55.494$, $p < .001$) failed to find main effects of sex, activity phase, or mating competency, all F values < 0.508 , p values $> .47$. A significant activity phase*mating competency interaction was found, $F_{(1,42)} = 4.342$, $p = .043$ and was

probably driven by an increase in relative neurogenesis from the early breeding condition (adjusted $M = 1.19\%$, $SE = .18$) to the early non-breeding condition (adjusted $M = 1.68\%$, $SE = .227$), but the post hoc analyses did not reach significance, $LSD p = .92$. No other interactions reached significance, all F values < 1.079 , p values $> .304$.

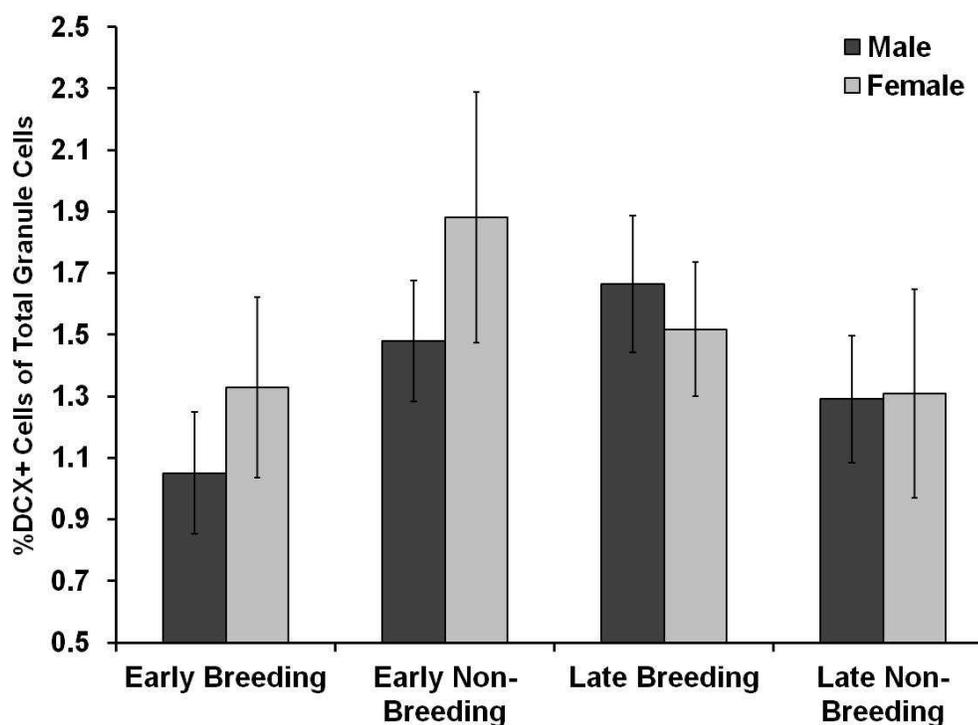


Figure 11. Mean (\pm SEM) numbers of DCX-positive cells relative to the total number of granule cells. Displayed means are corrected for lens weight. No effects of sex, mating competency, or activity phase were found. However, an activity phase*mating competency interaction was found whereby relative neurogenesis was lower during early breeding than during early non-breeding.

Granule Cell Estimates

Sex and Seasonal Analysis. Estimates of the total number of granule cells in each group are shown in Figure 12. No correlation was found between granule cell number and

absolute DCX estimates $r_{(51)} = .05$, $p = .73$, but granule cell number was negatively correlated with relative DCX estimates in a 1-tailed analysis, $r_{(51)} = -.25$, $p = .036$. A 2x2x2 ANOVA using the same subject variables as the above analyses failed to find main effects of sex, activity phase, or mating competency, all F values < 2.988 , p values $> .09$, or any significant interactions, all F values < 1.22 , p values $> .277$. Additionally, neither body weight ($F_{(1,42)} = 1.095$, $p = .301$) or lens weight ($F_{(1,42)} = .714$, $p = .403$) were significant covariates, so no further analyses were conducted to control for age effects.

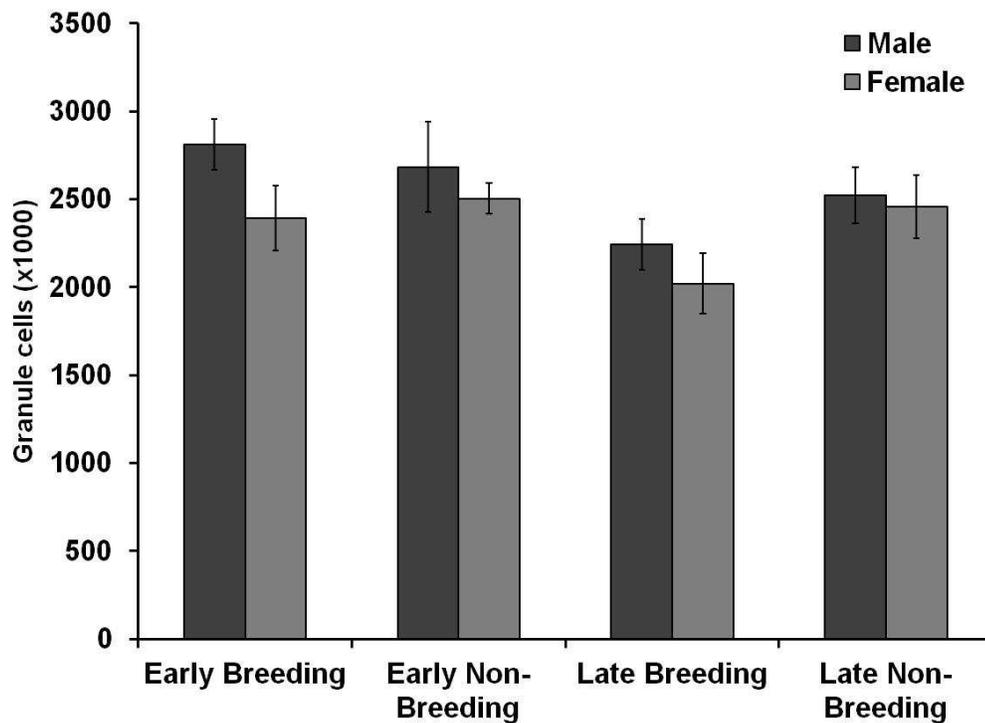


Figure 12. Mean (\pm SEM) number of total granule cells in males and females. The number of granule cells did not differ between sex, season, or mating competency. Additionally, neither body weight or lens weight were significant covariates.

Dentate Gyrus Volume

Sex and Seasonal Analysis. The mean DG volume for each group is shown in Figure 13. A weak positive correlation was found between DG volume and absolute estimates of DCX-positive cells that was significant in a 1-tailed analysis, $r_{(51)} = .24$, $p = .045$, but no correlation was found between DG volume and relative DCX estimates, $r_{(51)} = .06$, $p = .684$. A 2x2x2 ANOVA using the same subject variable as the above analyses revealed a significant main effect of mating competency, $F_{(1,43)} = 5.648$, $p = .022$, where DG volume was lower in the breeding condition ($M = .349 \text{ mm}^3$, $SE = .012$) than in the non-breeding condition ($M = .395 \text{ mm}^3$, $SE = .015$). The analysis failed to find main effects of sex or activity phase, F values < 3.888 , p values $> .054$, or any significant interactions, all F values $< .37$, p values $> .52$. Additionally, neither body weight ($F_{(1,42)} = .045$, $p = .832$) or lens weight ($F_{(1,42)} = .506$, $p = .481$) were significant covariates, so no further analyses were conducted to control for age-related effects.

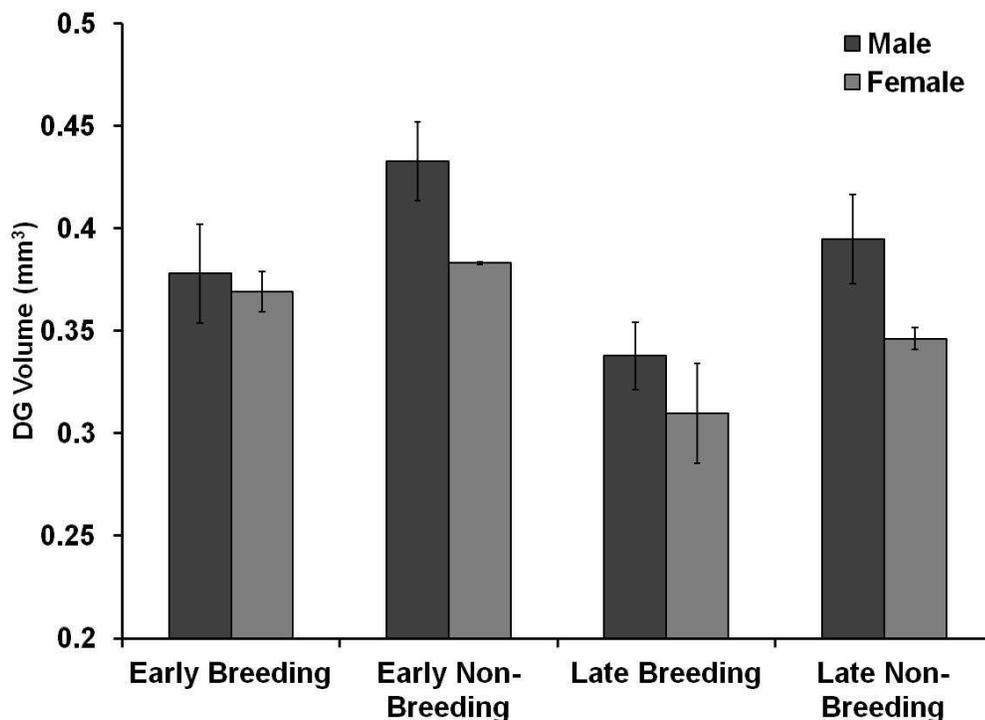


Figure 13. Mean (\pm SEM) volume of the DG in males and females. No sex differences were found, but DG volume was found to be significantly lower during breeding than non-breeding. Additionally, neither body weight or lens weight were significant covariates.

Discussion

This is the first experiment known to the author that has demonstrated the presence of adult neurogenesis in the eastern chipmunk, although this phenomenon has previously been observed in some other related species of chipmunk (Barker et al., 2005; Pan et al., 2013). With respect to sex and seasonal differences in neurogenesis, it was predicted that neurogenesis should be greatest in breeding males, when they increase their home range size and thus have the greatest requirement for spatial memory capacity. After normalizing DCX estimates to the total number of granule cells and controlling for age, analyses revealed that during the early activity phase, relative neurogenesis was lower during

breeding than non-breeding, opposite to the hypothesis that neurogenesis would increase in males during breeding. No difference between breeding and non-breeding was found in the late season, nor was there any sex difference. Thus, the analysis failed to find any evidence that neurogenesis is correlated with home range size in chipmunks. No sex or seasonal differences in total granule cell number were found, nor was the number of granule cells correlated with age. An effect of mating competency, but no sex difference, was found with DG volume, which is discussed below.

The lack of a sex difference in neurogenesis during breeding is contrary to some previous studies in wild rodents (Burger et al., 2014; Galea & McEwen, 1999), but in agreement with at least one (Lavenex et al., 2000b). This result is somewhat surprising given that sex differences in space use occur during breeding (Bowers & Carr, 1992; Elliott, 1978), presumably leading to greater spatial memory requirement for males in addition to the establishment of new spatial memories for their expanded home range. Indeed, new spatial learning increases neurogenesis in the laboratory (Ambrogini et al., 2000; Epp et al., 2010; Gould et al., 1999). With respect to seasonal fluctuation, relative neurogenesis was found to be lower breeding than non-breeding after controlling for age. This result is similar to some previous reports (Burger et al., 2014; Galea & McEwen, 1999), although the difference only occurs during the early activity phase. However, the result directly contradicts the prediction that neurogenesis should increase in males during breeding. Present results included, there appears to be no evidence to date that sex or seasonal fluctuation in neurogenesis is related to home range size (Burger et al., 2014; Galea & McEwen, 1999; Lavenex et al., 2000b).

Given that range size differences do not explain the seasonal fluctuation in neurogenesis in the present study, several other explanations may apply. Factors that influence rates of hippocampal neurogenesis such as physical activity (van Praag et al., 1999), gonadal hormone levels (Galea, 2008), sexual experience (Leuner et al., 2010), stress (Brummelte & Galea, 2010; Gould et al., 1997; Wong & Herbert, 2006), and changes in the intensity of food-caching (Pan et al., 2013) were not directly measured. One possibility is that neurogenesis during the early breeding season is low due to the aftereffects of hibernation. Spring breeding is the first activity that chipmunks engage in after emerging from hibernation (Elliott, 1978; Smith & Smith, 1972), and hibernation is known to cause a substantial decrease in neurogenesis (Popov et al., 2011). Neurogenesis may also be decreased during breeding by stress hormones, which peak during breeding in some animals (Boonstra, Hubbs, et al., 2001; Eggermann et al., 2013), a possibility suggested by other authors (Burger et al., 2014). If this were the case, it would be expected to have occurred during both breeding seasons, although the summer breeding season in chipmunks has been found to be somewhat inconsistent in previous field reports (Elliott, 1978; Pidduck & Falls, 1973; Smith & Smith, 1972).

Environmental factors may also explain the present results. Although there was no statistically-significant decrease in neurogenesis in the fall, the data resemble a curvilinear function, with neurogenesis rates peaking in the middle of the summer. This pattern is reminiscent of changes in photoperiod and temperature. Additionally, neurogenesis may have been affected by seasonal changes in food availability. For instance, chipmunks may have engaged in increased food-caching in order to exploit a particular food source which was transiently available (Elliott, 1978; Humphries et al., 2002). Indeed, a previous study

has demonstrated that the amount of food caching is correlated with neurogenesis in siberian chipmunks (Pan et al., 2013). It is also possible, perhaps even expected, that several of these factors interact to affect neurogenesis. An increase in food caching would necessarily be accompanied by an increase in physical activity, which also increases neurogenesis (van Praag et al., 1999). Similarly, longer photoperiod could possibly lead to greater physical activity given that chipmunks are diurnal (Snyder, 1982) and might then be more active during longer days.

The present results are consistent with the body of evidence that hippocampal neurogenesis decreases with age (Amrein et al., 2011, 2004; Barker et al., 2005; Epp et al., 2009; Kuhn et al., 1996). Although the absolute age of the chipmunks in the current study are not known, several indirect measures to estimate relative age negatively correlated with neurogenesis. Among the variables considered in the present experiment, including sex and season, these age measures seemed to be particularly strong predictors of neurogenesis. Although examination of the upper molars suggested that male chipmunks in the late non-breeding season were all "adults", these males had higher average neurogenesis than any other group in the study. Since this statistically-significant increase in neurogenesis was attenuated by the inclusion of age covariates such as body weight and lens weight, a likely explanation is that age-related decreases in neurogenesis in eastern chipmunks continue beyond the acquisition of adult molars.

It is, however, unclear why only males exhibited a fall increase in neurogenesis independent of age differences. Interestingly, a study by Pan et al. (2013) found that in siberian chipmunks, neurogenesis was correlated with scatter-hoarding intensity only in males, which suggests that, food caching being equal between sexes, there could still be a

sexual dimorphism in the responsiveness of neurogenesis to changes in food-caching behaviour. Thus multiple factors may have interacted with age effects or acted independently to lead to a sex and seasonal difference in neurogenesis, and conclusions relying on indirect measures of age should be made with caution.

The present study found no evidence of sex differences in DG volume, which is surprising given the sex difference in HPC volume reported earlier in Experiment 1 as well as the fact the male chipmunks have larger range sizes during breeding (Bowers & Carr, 1992; Elliott, 1978), which should lead to a larger male DG. Furthermore, there was a seasonal difference in DG volume in which DG volume was higher in the non-breeding season, also contradicting seasonal trends in space use, but consistent with the absolute HPC volume findings of Experiment 1. However, two consecutive studies in Richardson's ground squirrels found contradictory results in DG volume, with a sex difference, but no seasonal difference, in one study (Burger et al., 2013), and a seasonal difference, but no sex difference, in the subsequent study (Burger et al., 2014). As Burger et al. (2014) argue, as well as the present thesis argues, wild-living animals undergo transient and unpredictable environmental pressures influencing the volume of brain structures outside the pressures related to their routine behavioural ecology. The present results may be a result of idiosyncratic seasonal changes in environmental conditions exerted on the present sample.

The findings of the present study highlight the importance of including as precise as possible a measure of age when comparing rates of neurogenesis in natural populations in which the age of captured individuals can vary significantly. Age surfaced as a very strong predictor of neurogenesis within the present results. Despite this, evidence of seasonal changes in neurogenesis was found even after controlling for age and granule cell number,

but the pattern of results is inconsistent with the view that fluctuations in range size drive fluctuations in neurogenesis. Rather, the present pattern of results may reflect the effects of hibernation, emphasizing the need to address this confound. Furthermore, no sex differences in neurogenesis were found, further disconfirming the hypothesized pattern. Thus, although the present results are evidence of age-independent, seasonal plasticity in hippocampal neurogenesis, they fail to confirm a strong link between neurogenesis and home range size and instead point to several other environmental or behavioural factors.

CHAPTER 4 - GENERAL DISCUSSION

The present study aimed to examine whether the HPC exhibits differences in a naturalistic setting in order to accommodate increased spatial memory requirement related to spatial behaviour. To this end, the HPC was examined in the eastern chipmunk, *Tamias striatus*, a species in which males increase their home range size during two breeding seasons each year, with minimal sex differences in spatial behaviour outside of breeding (Bowers & Carr, 1992; Elliott, 1978; Smith & Smith, 1972). Two main analyses were conducted: 1) Examining HPC volume across sex and season, including the absolute volume of the HPC and HPC volume relative to overall brain volume, and 2) Examining rates of hippocampal neurogenesis across sex and season, including the absolute number of immature granule cells, the proportion of immature granule cells to total granule cells, and the volume of the DG. Both experiments also examined relative age, as measured by body weight and eye lens weight, as a cofactor in each analysis. Ultimately, lens weight was considered the most accurate age variable due to its independence of differences in food intake. I found both sex and seasonal differences in the eastern chipmunk HPC. The HPC was found to be larger in males than in females after controlling for age and total brain

volume, but did not differ between breeding and non-breeding periods. Adult hippocampal neurogenesis, which had not been previously confirmed in eastern chipmunks prior to the present work, did not exhibit any pronounced sex difference after controlling for age and normalizing cell counts to the total number of neurons in the DG, but was found to be seasonally-variable, increasing after the early breeding season and then remaining stable across subsequent seasons.

When considered in the context of previous literature examining sex and seasonal differences in the HPC of wild species (Burger et al., 2014, 2013; Clayton et al., 1997; Galea & McEwen, 1999; Lavenex et al., 2000a, 2000b), the present findings support the view that the HPC is sexually and seasonally variable under natural conditions. However, the specific pattern of results, as discussed in the context of each experiment, do not completely map onto sex or seasonal differences in eastern chipmunk behavioural ecology in an obvious way, nor do these findings fully corroborate the pattern of results in previous studies (Burger et al., 2014, 2013; Clayton et al., 1997; Galea & McEwen, 1999; Lavenex et al., 2000a, 2000b). Although relative HPC volume was greater in males, consistent with their greater space use during breeding, no variation was seen in relative HPC volume between breeding and non-breeding conditions. Neurogenesis exhibited neither sex nor breeding-related differences that correlated with space use. Thus, the present study found no evidence that seasonal plasticity in the HPC correlates with increased spatial memory demand.

The present study was designed to overcome several confounds present in previous research (Burger et al., 2014, 2013; Clayton et al., 1997; Galea & McEwen, 1999; Lavenex et al., 2000a, 2000b). Eastern chipmunks have two breeding seasons, one of which is

during midsummer (Elliott, 1978; Pidduck & Falls, 1973; Smith & Smith, 1972), enabling the analysis of the HPC during a breeding season distal to emergence from hibernation. Chipmunks also exhibit no sex differences in food caching behaviour (Elliott, 1978). Furthermore, chipmunks are relatively long-lived, with a lifespan of 2-3 years (Tryon & Snyder, 1973), and the present analysis additionally controlled for age using eye lens weight as a continuous age covariate, an extremely important feature given that both HPC volume and neurogenesis correlate with age (Epp et al., 2009; Kuhn et al., 1996; Perrot-Sinal, Kavaliers, & Ossenkopp, 1998). Having thus isolated the effects of fluctuating home range size in males from several confounding factors, the present results suggest that changes in range size are not sufficient to drive seasonal fluctuations in HPC volume or neurogenesis.

The findings are surprising given that spatial learning causes increases in both HPC volume (Scholz, Allemang-Grand, Dazai, & Lerch, 2015) and neurogenesis (Ambrogini et al., 2000; Epp et al., 2010; Gould et al., 1999) in the laboratory. However, the naturalistic setting from which chipmunks were captured may essentially represent a maximally enriched environment. As such, both HPC volume and neurogenesis may be at ceiling, exhibiting no obvious seasonal fluctuations related to differential spatial memory requirement. With respect to the observed sex differences in HPC volume, male chipmunks may then simply have a larger HPC than females at baseline in order to allow greater flexibility to increase their range size.

Studies within the same species do not always yield the same findings from year to year or sample to sample. For example, Burger et al. (2014) and Burger et al. (2013) each used Richardson's ground squirrels captured from different field seasons and found

different results in each study in the effects of sex and season on total brain volume and DG volume. Similarly, seasonal variation of HPC volume in black-capped chickadees are not consistently observed across studies (Hoshooley et al., 2007; Smulders et al., 1995). Differences such as these that are not necessarily related to routine seasonal change may be a result of factors such as environmental stress or water content (Frodl & O'Keane, 2013; Pucek, 1965; Weinstock, 2011).

Unpredicted differences in behaviour may also contribute to variation between studies. Chipmunks, though being understood to have two breeding seasons, are not always observed to mate during the summer breeding season in every year of observation (Elliott, 1978; Smith & Smith, 1972). Additionally, the temporal boundaries of breeding seasons can be enigmatic and differ between populations or geographic regions (Elliott, 1978; Pidduck & Falls, 1973; Smith & Smith, 1972). The present study relied on delineations of breeding seasons from previous observations in a relatively nearby area with the same type of climate and vegetation (Smith & Smith, 1972), providing relative confidence that the current study sample would have had similarly-timed behaviours. However, I do not know with certainty whether chipmunks in the present sample conformed to my temporal delineations of breeding seasons. Moreover, the timing and length of hibernation may be affected by the size and quality of food stores (Humphries et al., 2003; Munro et al., 2005) as well as temperature patterns (Elliott, 1978; Yahner & Svendsen, 1978). In a given year, chipmunks do not necessarily enter or emerge from torpor at a prescribed time. Space use may also vary considerably based on food and water availability (Bowers, Welch, & Carr, 1990; Elliott, 1978; Forsyth & Smith, 1973; Mares, Watson, & Lacher, 1976), the presence

of competitors (Giraldeau, Kramer, Deslandes, & Lair, 1994), and idiosyncratic variability in home range use among individuals (Getty, 1981a).

Thus, multiple environmental factors that change across years and geographic locations can affect chipmunk behaviour. If the morphology and neurogenic rate of the HPC is connected to behaviour, these unpredicted changes might affect sex and seasonal variation of the HPC. Nonetheless, the variability between studies within the same species (Burger et al., 2014, 2013; Hoshooley et al., 2007; Hoshooley & Sherry, 2004; Smulders et al., 1995) may still be of valuable insight, in that it certainly suggests that under natural conditions, the HPC is influenced by much more than the routine sex and seasonal differences that are typically considered when forming neuroecological predictions about the HPC.

One important caveat with the present study that should be noted is that it is completely correlational. No direct observations or manipulations were made with specific behaviours such as food caching, mating, or general levels of physical activity. Of course, nor were any manipulations conducted on environmental factors such as daily photoperiod, temperature, food-availability, or predation, either. Therefore, no conclusions can be made about the causal relationships underlying seasonal and sex differences in the HPC in these data. This leaves open the question of whether seasonal cues such as photoperiod or temperature signal the HPC to make plastic changes such as an up- or down-regulation of neurogenesis in preparation for a given season's cognitive requirements, or if such changes in the HPC are driven by chipmunks adapting their behaviour to the survival or reproductive demands in a given season. It would be feasible, in principle, to account for many of these variables by conducting radio telemetry to measure physical activity and

ranging behaviour, assay tail blood samples for hormone levels, and examine stomach content or surrounding food sources to assess food availability. However, this would only provide more control variables and axes for statistical comparisons, and would not necessarily provide any more logical basis for drawing conclusions about causality.

Additionally, the present analyses might have been affected by uneven representation of sex and age in the present sample. The female sample was smaller than the male sample, with only two females collected during the month of May. This was partially caused by the fact that many females are pregnant or lactating after breeding seasons (Pidduck & Falls, 1973), which seemed to be the case in the present study and was a criterion for excluding them, leading to the exclusion of two females that could otherwise have been included. A larger female sample would have contributed additional statistical power and possibly reflected a greater part of the scope of individual variability within females. However, the present sample was of sufficient power to reveal both sex and seasonal differences in HPC volume and neurogenesis, so the paucity of females did not completely hinder these comparisons.

Another important methodological consideration in the present study is that the conclusions were arrived at based on relative measures of HPC volume and neurogenesis that were age-corrected using lens weight. Considering absolute HPC volume or neurogenesis, as well as controlling for age using body weight rather than lens weight, led to different results. However, relative measures age-corrected with lens weight were arguably the most accurate and representative measures upon which to form conclusions. Absolute HPC volume can differ between individuals due to gross differences in brain size without reflecting how much of the brain's metabolic resources are devoted to it. Absolute

neurogenesis can differ between individuals simply as a function of some individuals having more or fewer cells in the DG without reflecting any differential investment in the generation of new neurons. Therefore, relative measures reflect the true amount of metabolic investment in HPC tissue and new neurons. With respect to age controls, lens weight was preferred as the most accurate age measure. For one, it is a continuous variable, meaning that it was able to be included as a covariate in the analyses and could distinguish fine age differences unlike dentition. It is also more accurate than body weight because the eye lens steadily accumulates weight across the lifespan (Augusteyn, 2014), whereas body weight can fluctuate due to food intake, a confound not affecting lens weight. As such, lens weight-controlled relative measures of HPC volume and neurogenesis were deemed to be the most accurate measures upon which

Analysis of lens weight suggested that age representation may be an important factor, with chipmunks captured during the fall being younger than those captured during the spring. This may have resulted from an increase in young chipmunks over the course of the active season, given that chipmunks captured during the spring were almost certainly overwintered adults born the previous year or earlier. Chipmunks born after the spring breeding season can reach adult body weight by the end of the summer (Pidduck & Falls, 1973), meaning that chipmunks captured during fall in the present sample may have been born during the same active season, despite exhibiting adult size at the time of capture. Age was controlled for during analysis, and most chipmunks met the dental criteria for mature adults. However, it has been suggested that examining animals within a year of their birth may lead to a confound whereby developmental processes are conflated with adult plasticity (Lavenex et al., 2000a). This remains a significant challenge in wild-living

animals that may perish across a hibernation season. Other than the finding that neurogenesis decreases, on average, across the lifespan (Amrein et al., 2011, 2004; Epp et al., 2009; Kuhn et al., 1996), including in chipmunks (Barker et al., 2005), with some fluctuation related to seasonal behaviours in some species (e.g. Burger et al., 2014; Galea & McEwen, 1999), it is not clear what developmental changes might occur in the chipmunk HPC after reaching adulthood. However, there might be behavioral differences during the first year of birth apart from physiological development that are distinct from subsequent years, such as dispersing place of birth to find their own burrows (Elliott, 1978). Hence, differences in behaviour between adults and juveniles may also be a confound when making predictions about the HPC based on natural behaviour.

A direct approach to answering causal questions about seasonal hippocampal plasticity that avoids problems such as unpredicted year to year differences and sex or age biases in trapping may be to conduct experiments within semi-naturalistic environmental enclosures, where many aspects of natural behaviour can be preserved and still allow the direct manipulation of environmental factors. One study in chipmunks has already taken this approach. Pan et al. (2013) examined the effects of scatter-hoarding intensity on hippocampal neurogenesis in a semi-naturalistic colony of siberian chipmunks and found that cell proliferation increased with the intensity of scatter-hoarding behaviour specifically in males. However, this study captured wild chipmunks of unknown age, meaning that age-related changes in neurogenesis could not be accounted for. Additionally, seasonal changes were not directly manipulated.

Other studies have taken a more controlled approach by manipulating photoperiod to signal seasonal changes. Photoperiod manipulation causes changes in spatial learning

and hippocampal dendritic morphology (Workman, Bowers, & Nelson, 2009), synaptic plasticity (Walton et al., 2011), and neurogenesis (Walton, Aubrecht, Weil, Leuner, & Nelson, 2014) in white footed-mice, as well as hippocampal dendritic morphology in siberian hamsters (Ikeno, Weil, & Nelson, 2013; Workman, Manny, Walton, & Nelson, 2011). However, none of these studies compared sexes or revealed any gross differences in HPC volume. When both sex and photoperiod length are compared, no differences in HPC volume are found, despite photoperiod-related differences in spatial memory (Galea et al., 1994; Krebs et al., 1995; Macdougall-Shackleton, Sherry, Clark, Pinkus, & Hernandez, 2003).

These results seem to indicate that photoperiodic differences across seasons can influence spatial memory and cause more subtle changes in the HPC, but gross seasonal changes in HPC volume have not been replicated under these conditions. Of course, photoperiod is far from the only environmental variable that changes with seasons, with additional factors being temperature and food-availability. Additionally, it is not known whether seasonal differences in the environment affect the responsiveness of the HPC to behavioural changes. Specifically, the HPC may exhibit a greater capacity for plasticity during the breeding season than during the non-breeding season, irrespective of whether overall anatomical differences are observed.

Ultimately, however, semi-naturalistic studies would still necessarily restrict the level of environmental enrichment and the scope of natural behaviour present in a fully naturalistic setting. For instance, it would be very difficult to provide a confined study area within which breeding-related increases in range size could occur naturally in a significant number of subjects. It may thus be more advantageous to continue working in wild-living

populations whilst conducting behavioural observations using radio telemetry and assaying steroid hormones. After all, it is the investigation of the correlation between the brain and naturalistic, evolved behaviours that is the goal of neuroecology (Sherry, 2006).

CONCLUSIONS

In this thesis, I have reviewed the neuroecological literature regarding the HPC in mammals and birds and have described the results of the present study concerning sex and seasonal variation in HPC volume and neurogenesis in the eastern chipmunk, *Tamias striatus*. I found evidence of both sex and seasonal differences in the HPC of wild-living eastern chipmunks, contributing to a presently sparse literature examining both sex and seasonal changes in the HPC of wild-living species. Predictions of sex and seasonal differences in the HPC based on the general knowledge of chipmunk behavioural ecology were partially confirmed by the present findings. However, there were unexpected findings that may be better explained by unpredicted differences in individual behaviour, age, the timing of seasonal behaviours, and environment. No seasonal fluctuation in either HPC volume or neurogenesis was found that correlated with breeding, when males increase their home range size, suggesting that changes in home range size do not drive seasonal change in the HPC.

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APPENDIX 1

Supplementary Figures

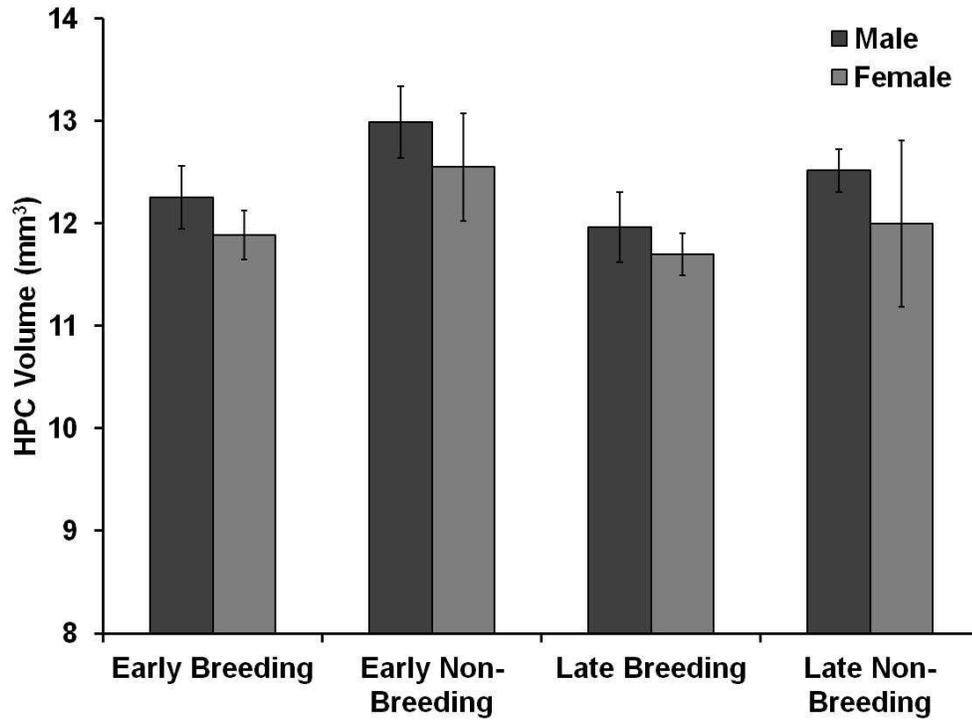


Figure 14. Mean (\pm SEM) absolute volumes of the HPC in male and female chipmunks. Displayed means are not corrected for age covariates, but males had significantly greater absolute HPC volume when both body weight or lens weight were included as covariates.

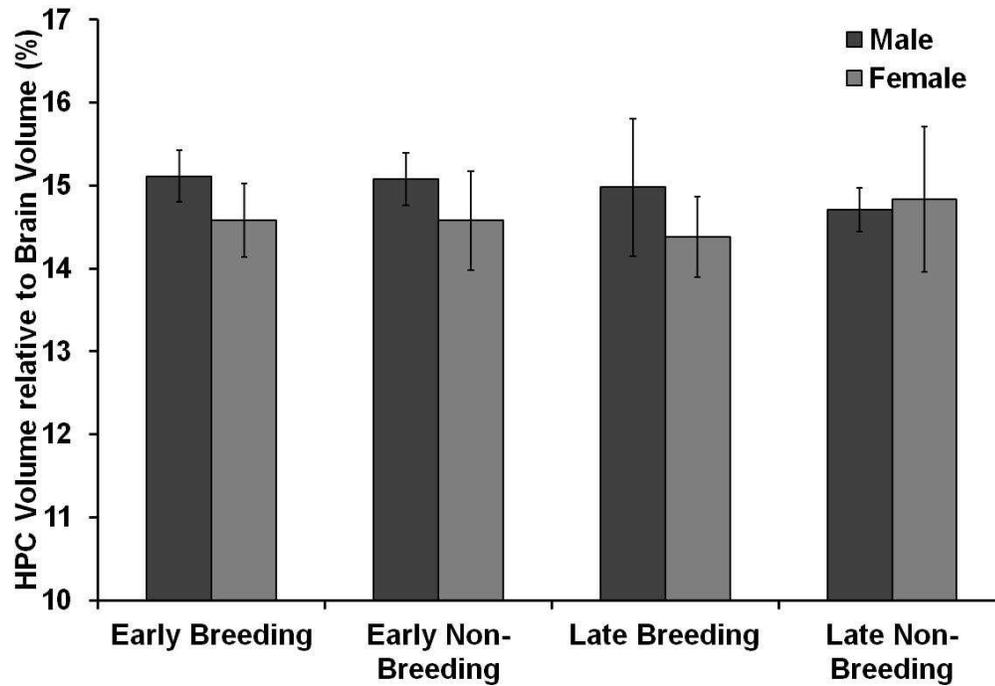


Figure 15. Mean (\pm SEM) proportions (%) of the HPC to surrounding tissue in males and females. Displayed means are not corrected for age, and no effects of sex, mating competency, or activity phase were found.

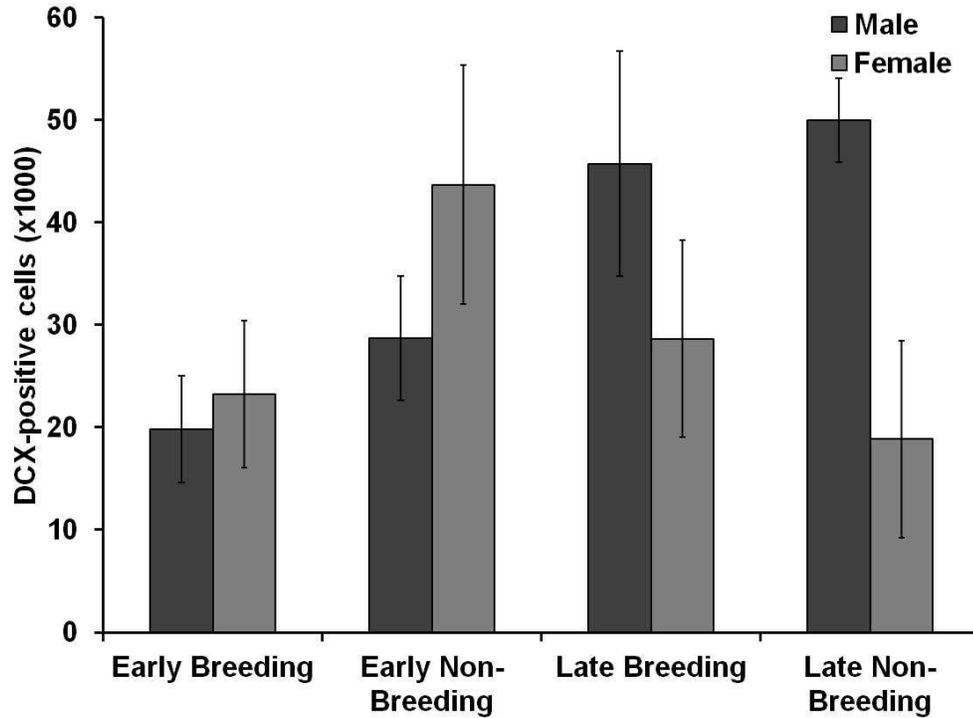


Figure 16. Mean estimates of DCX-positive cells (\pm SEM). Displayed means are uncorrected for age covariates. Including body weight or lens weight as covariates did not reveal sex differences in neurogenesis. A seasonal increase in males from spring to fall, as well as an increase from breeding to non-breeding, was found when using body weight as a covariate, but no differences were found when using lens weight.

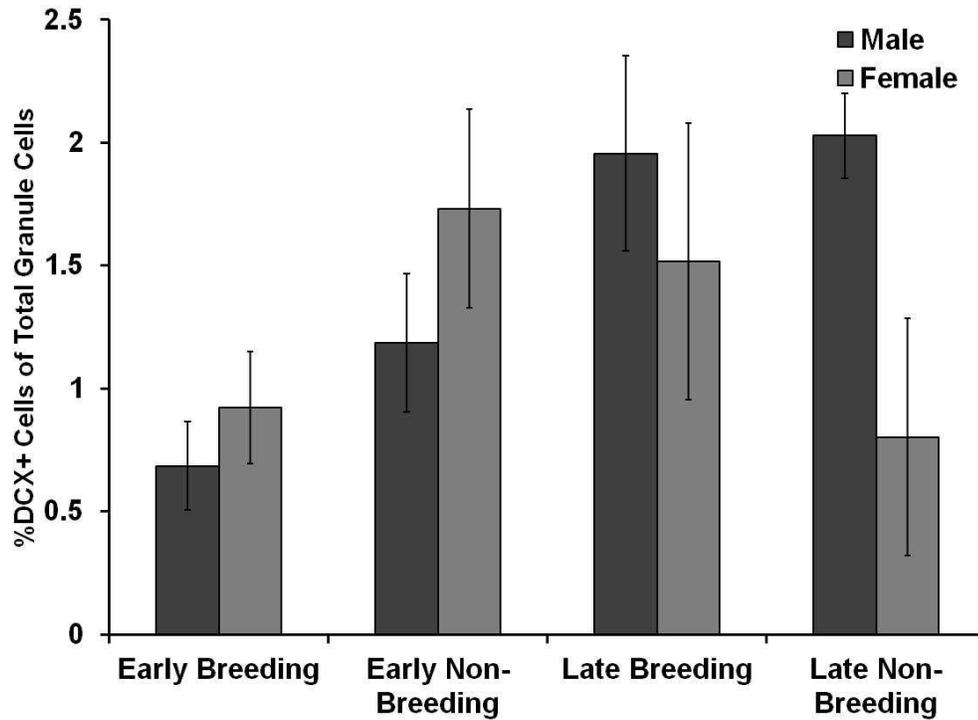


Figure 17. Mean (\pm SEM) numbers of DCX-positive cells relative to the total number of granule cells. Displayed means are not corrected for age covariates. Including either body weight or lens weight as covariates revealed no sex difference in relative neurogenesis, but did reveal an increase from breeding to non-breeding. This effect was specific to the spring when using lens weight as a covariate.