NATURAL PHOTOPERIODIC REGULATION OF HIPPOCAMPAL NEURAL PLASTICITY IN WILD MALE AND FEMALE BLACK-CAPPED CHICKADEES (*POECILE ATRICAPILLUS*)

by

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ABSTRACT

Food storing, a hippocampus-dependent spatial behaviour, fluctuates seasonally in black-capped chickadees (*Poecile atricapillus*), who store more food in the fall months for later retrieval in the resource-scarce winter. Supporting this behavioural plasticity is hippocampal neural plasticity. However, previous studies on how the hippocampus changes seasonally are inconsistent; it appears captivity may contribute to this variability. Here, I examined seasonal patterns of hippocampal plasticity in wild, non-captive male and female black-capped chickadees (N=36 adults). While hippocampal plasticity in males was minimal, female chickadees captured in winter (December-January) had increased hippocampal neurogenesis (measured using doublecortin immunoreactivity) compared to females captured in March-April and August-September. While sex differences in brain and behaviour are observed in some avian and non-avian species, these data are the first evidence of sex differences in the hippocampus of black-capped chickadees, and suggest there may winter-specific physiological and social pressures borne only by females which require specialized neural infrastructure.

Keywords: hippocampus, neural plasticity, neurogenesis, sex differences, black-capped chickadee

LIST OF ABBREVIATIONS AND SYMBOLS USED

[^{3}H]-TdR	tritiated thymidine
AHY	after-hatch-year
BrdU	5-bromo-2'-deoxyuridine
ca.	circa (approximately)
CI	confidence interval
CORT	corticosterone
DAB	3,3'-diaminobenzidine
DCX	doublecortin
DCX+	DCX-stained
DCX-ir	DCX immunoreactivity
%DCX+ coverage	proportion of DCX-ir per microscope FOV
DNA	deoxyribonucleic acid
E2	17β-estradiol
FOV	field of view
FSH	follicle stimulating hormone
GnRH	gonadotropin-releasing hormone
HA	hyperpallium apicale
Hp	hippocampus
HP1	dorsolateral portion of the hippocampus (sampling frame)
HP2	ventromedial portion of the hippocampus (sampling frame)
HPA	hypothalamic-pituitary-adrenal
HPG	hypothalamic-pituitary-gonadal
HY	hatch-year
IgG	Immunoglobulin G
LH	luteinizing hormone
LNP	lateral nidopallium
NRC	National Research Council
p_B	Bonferroni-adjusted p-value
PBS	phosphate-buffered saline
PFA	paraformaldehyde
T	testosterone
TBS	Tris-buffered saline
TBS-T	0.1% Triton-X in TBS
RA	robust nucleus of the arcopallium
RFID	radio frequency identification
ROI	region of interest
SVZ	subventricular zone
≈	approximately equal to
≪	much less than
≫	much greater than

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CHAPTER 1: GENERAL INTRODUCTION

Across the temperate zone, many vertebrates are forced to adapt their behaviours in response to changes in the environment. How this behavioural plasticity is manifested in the neural structures supporting these changing behaviours is of particular interest to neuroethologists and biologists alike. Further, many of the mechanisms underlying changes in the brain (or neural plasticity), particularly in non-traditional animal models including songbirds (order Passeriformes), have not been fully elucidated, and therefore remain relatively unclear.

The primary objective of this study was to determine whether there was seasonal plasticity in the hippocampus of a food-storing bird, the black-capped chickadee (*Poecile atricapillus*), by quantifying both the volume of and the number of new neurons in the hippocampus in birds captured at different times of the year. Secondary objectives were to investigate potential sex differences in hippocampal plasticity, and to address inconsistency among results of previous studies due to number of subjects and varying length and types of captivity through capturing wild male and female chickadees in larger numbers per group (n = 8 of each sex) and eliminating captivity in the experimental design.

1.1. PHOTOPERIODIC REGULATION OF BREEDING IN BIRDS

Many bird species in the temperate zone are seasonal breeders (Sharp, 1984), meaning that reproduction is restricted to a certain period of the year (typically when resources are abundant and climatic conditions are favorable) to maximize the likelihood of offspring survival (Nakao et al., 2008). Photoperiod (or day length) is the primary regulator of seasonal breeding in temperate birds via its effects on the hypothalamic-pituitary-gonadal (HPG) axis (reviewed in Ball, 1993; Dawson et al., 2001; Sharp, 2005; Rose et al., 2022). Briefly, as day length increases (as in spring), birds enter a breeding state known as photostimulation, resulting in increased secretion of gonadotropin-releasing hormone (GnRH) from the hypothalamus. This eventually leads to gonadal maturation and increased release of gonadal steroid hormones via its downstream effectors, follicle stimulating hormone (FSH) and luteinizing hormone (LH). Gonadal steroids, including testosterone (T) and 17β-estradiol (E2) are at maximal concentrations

in photostimulated birds, targeting various tissues to induce the physiological and behavioural changes necessary for successful breeding (Rose et al., 2022). Consistent long days (as in summer) induces photorefractoriness, in which the HPG axis no longer becomes responsive to changes in day length; this ultimately leads to gonadal regression, with some considering photorefractoriness analogous to a pre-pubertal state (Dawson et al., 2001). Finally, exposure to short days (as in fall/winter) induces a breeding state known as photosensitivity, in which the HPG axis is effectively reset, allowing birds to once again become responsive to changes in photoperiod (Dawson and Sharp, 2007).

1.2. SEASONAL CHANGES IN SONGBIRD BRAIN AND BEHAVIOUR – EVIDENCE FROM BIRDSONG

Of all the behavioural differences observed on a seasonal basis in birds, perhaps the most noticeable is the change in vocal behaviour. Male songbirds typically sing more elaborate, complex songs during breeding season to attract mates (Nottebohm, 1975). Song output increases in breeding season across many species, and while birds do sing during the post-breeding and non-breeding periods, it is at lower rates relative to the breeding season (reviewed in Ball, 1999; Gil and Gahr, 2002). Consequently, these seasonal changes in vocal behaviour lead to seasonal change in the nuclei of the vocal control system, a series of interconnected structures and circuits in the songbird brain that support the perception, learning and production of vocalizations (reviewed in Brenowitz et al., 1997; Brenowitz, 2004; Rose et al., 2022). For example, motor nuclei HVC (letter-based proper name) and robust nucleus of the arcopallium (RA) are significantly larger in breeding males than in non-breeding males (e.g., Smith et al., 1995; Smith, 1996). Further, in species in which only males sing, there is a marked sexual dimorphism in the size of HVC and RA, with these structures being virtually non-existent in females (reviewed in MacDougall-Shackleton and Ball, 1999). The peak in gonadal steroids (primarily T) as a result of photostimulation (described above) is generally considered the primary physiological and endocrine regulator of the seasonal neuroanatomical change observed in the vocal control system (reviewed in Smith et al., 1997; Tramontin and Brenowitz, 2000). Further, manipulations of photoperiod in both laboratory-based (i.e., experimental studies of birds housed in captivity) and field-based (i.e., observational and

correlational studies of birds captured at different times of year) studies reliably induce changes in these vocal control structures across a wide range of songbird species (reviewed in Brenowitz, 2004).

1.3. ADULT NEUROGENESIS

Neurogenesis is the multi-step process through which new neurons are formed in the brain and become integrated into structures and circuits within the central nervous system (Zhao et al., 2008; Gage, 2019). This process can largely be divided into four main stages: proliferation, differentiation, migration, and survival (Barnea and Pravosudov, 2011). Proliferation describes the production of new cells from neural stem cells or neural progenitors (Doetsch and Scharff, 2001; Gonçalves et al., 2016); these cells then differentiate into various neuronal or glial phenotypes. Young neurons are then recruited to specific brain areas and migrate to their final destination (via radial glia), where they mature and incorporate into circuits and systems, replacing older, dying neurons (Barnea and Pravosudov, 2011; Pytte, 2016). In the avian brain, neuronal progenitors are born in the subventricular zone (SVZ) of the lateral ventricle (Goldman and Nottebohm, 1983; Alvarez-Buylla et al. 1990) and migrate throughout the telencephalon (Alvarez-Buylla and Nottebohm 1988; Vellema et al. 2010), not only during development, but also throughout adulthood.

For many years, it was believed that neurogenesis was restricted exclusively to the developmental period and did not occur throughout adulthood; indeed, the notion of the adult brain being "fixed and immutable" was first proposed by the father of modern neuroscience, Santiago Ramón y Cajal in 1928, and remained the "central dogma of neurobiology" until well into the 1970s and 1980s (reviewed in Colucci-D'Amato et al., 2006; Owji and Shoja, 2020). The initial discovery of adult neurogenesis is generally attributed to Josef Altman, who unexpectedly found that injecting tritiated thymidine (a marker of cell proliferation) into lesioned areas of the brain of adult rats (*Rattus norvegicus* var. *domesticata*) led to labelling of neurons and neural progenitors in non-lesioned areas (Altman, 1962). Altman (1963) subsequently replicated these findings with an experimental study, showing that injection of the same cell birth marker in adult rats and cats (*Felis catus*) led to labelling of neurons in both the dentate gyrus and the

cerebral cortex. However, Altman's discoveries remained generally unaccepted by the larger scientific community for the better part of the next 20 years (Gross, 2009; Owji and Shoja, 2020).

It was not until the pioneering work in canaries (*Serinus canaria* var. *domesticata*) completed by Fernando Nottebohm and colleagues in the 1980s that the larger community became more accepting of the prospect of adult neurogenesis (Owji and Showa, 2020). Goldman and Nottebohm (1983) first showed that injecting adult canaries with tritiated thymidine resulted in pools of labelled neurons throughout the telencephalon, including SVZ and motor nucleus HVC, when birds were sacrificed 3-5 weeks after injection. Later studies then proved that new neurons in the songbird brain become recruited and functionally integrated into existing neural regions and circuits (Paton and Nottebohm, 1984), including the circuit known as the descending motor pathway between HVC and RA (Alvarez-Buylla et al., 1990). In short, these seminal studies in songbirds forced the broader scientific community to rethink its hesitancy in supporting the notion of adult neurogenesis in mammals and primates (e.g., Gould and McEwen, 1993; Gould et al., 1999). Since then, the presence of adult neurogenesis has been confirmed and validated in rodents, non-human primates, humans, and other vertebrates (reviewed in Gould, 2007; Bonfanti and Peretto, 2011; Jessberger and Gage, 2014). However, unlike in rodents and primates, where adult neurogenesis is generally restricted to a select few brain regions (e.g., subventricular zone, olfactory bulb, dentate gyrus of the hippocampus, striatum; reviewed in Bergmann et al., 2015; Snyder, 2019; Lucassen et al., 2020), songbirds remain one of the few vertebrate models (in addition to some fish and reptile species; reviewed in Chapouton et al., 2007) where adult neurogenesis occurs throughout the telencephalon.

1.4. QUANTIFYING NEURAL PLASTICITY

There are multiple approaches through which neural plasticity can be examined and quantified. In the context of studies of animal models (including birds), such approaches typically involve processing and examining post-mortem brain tissue using a variety of standard histological and stereological techniques (reviewed in Mayhew, 1992). In birds, studies examining systems-level neural plasticity (i.e., changes in entire

brain regions and circuits) usually take one of three approaches: quantifying overall differences in region volume, neuron number, or neurogenesis.

Differences in region volume and neuron number are generally examined by staining brain tissue with a histological dye that allows for the visualization of neurons (Mayhew, 1992). For example, cresyl violet and thionine are both commonly used, basic histological dyes that stain DNA and Nissl substance (rough endoplasmic reticulum and ribosomes; Kádár et al., 2009). The high amount of protein synthesis that occurs in neurons (and therefore increased concentrations of Nissl substance in the cytoplasm) make these dyes particularly effective at staining neurons. However, these dyes are non-selective and also label non-neuronal glia (García-Cabezas et al., 2016). An alternative to these non-specific histological dyes is to apply traditional immunohistochemical techniques (i.e., using antibodies to label particular antigens of interest) to stain for proteins expressed specifically in mature neurons, including neuronal nuclear protein (NeuN; Duan et al., 2016).

Once brain tissue is stained, it can be examined under a microscope where the size of a particular region across serial tissue sections can be measured (usually by tracing the region's area); these area measurements can then be used to calculate region volume using a variety of stereological estimating techniques (reviewed in Rosen and Harry, 1990; Mayhew, 1992). Similarly, by counting the number of neurons within a small sampling frame over several sections; one can use one of several well-established extrapolation approaches (reviewed in Mayhew, 1992; Schmitz and Hof, 2005) to estimate overall neuron numbers within a region.

Quantifying neurogenesis in the brain generally requires the labelling of either exogenous or endogenous markers of new or immature neurons. Initial studies of neurogenesis in mammals and birds (e.g., those previously described; Altman, 1962; Altman, 1963; Goldman and Nottebohm, 1983, etc.) relied on exogenous markers such as tritiated thymidine ([^{3}H]-TdR), a radioactive nucleotide analog, to quantify cell proliferation. Using this approach, [^{3}H]-TdR is injected into the subject; some portion of the radiated nucleotides are then incorporated into newly dividing cells during the S phase of the cell cycle (at the time of DNA replication; reviewed in Cavanagh et al., 2011). Following sacrifice and sectioning, cells with [^{3}H]-TdR in the nucleus (detected

using autoradiography) are therefore cells which had divided between the time of injection and sacrifice. The same technique and theory apply to quantifying neurogenesis using synthetic thymidine analogs, including 5-bromo-2'-deoxyuridine (BrdU), considered the "gold standard" approach to quantifying neurogenesis (Wojtowicz and Kee, 2006; Balthazart and Ball, 2014a).

An alternative to using exogenous markers of neurogenesis is to apply traditional immunohistochemical techniques to target proteins or factors expressed only in immature and/or newly divided neurons (e.g., Balthazart and Ball, 2014a; Kuhn et al. 2016). Such an approach is advantageous as it does not require administering potentially harmful chemical labels (usually over multiple injections) to animals while they are still alive; indeed, both [^{3}H]-TdR and BrdU are cytotoxic and have been shown to have harmful, mutagenic, and potentially confounding effects on neurogenesis in mammals and birds (e.g., Hu et al., 2002; Taupin, 2007; Balthazart and Ball, 2014a).

One such protein, doublecortin (DCX), has been well-used as an endogenous marker of immature neurons in studies of adult neurogenesis in vertebrates including rodents (e.g., Francis et al., 1999; Brown et al., 2003; Couillard-Despres et al., 2005), birds (e.g., Boseret et al., 2007; Balthazart et al., 2008), reptiles (e.g., LaDage et al., 2013; LaDage et al., 2017; Ngwenya et al., 2018), and teleost fish (e.g., Tozzini et al., 2012). DCX is a microtubule-associated protein that is expressed specifically in immature neurons for up to approximately a month post-mitosis (Brown et al., 2003; Balthazart and Ball, 2014a; Balthazart and Ball, 2014b). In birds, DCX is a valid and reliable marker of neurogenesis (Balthazart and Ball, 2014b) and has been used extensively to quantify neural plasticity both in a variety of species and in a variety of brain regions. A further advantage of DCX is that due to its cellular role as a cytoskeleton-associated protein, staining for DCX allows for the visualization of the entire neuron, as opposed to BrdU, which only visualizes the cell nucleus (Taupin, 2007); therefore differences in the morphologies of immature neurons can be examined. Various morphologies and cell classification schemes for DCX-stained (DCX+) neurons have been described and proposed (e.g., Balthazart et al., 2008); however, to date, there is no standardized cell classification schemes or nomenclature to describe DCX+ cells in the songbird brain,

perhaps because the specific morphologies of DCX+ cells may vary between brain regions and across species (e.g., Hall et al., 2014a).

1.5. STUDY SPECIES

The species of interest studied in this thesis is the black-capped chickadee (*Poecile atricapillus*). Black-capped chickadees are a non-migratory North American songbird whose range extends from Alaska in the northwest to the Appalachians in the southeast (Foote et al., 2020). Across its range, breeding season is generally confined to between April and June (but can extend as late as July), although the fine-scale timelines (e.g., egg laying, hatching) vary with climate and region and can also vary across years (reviewed in Ramsay and Otter, 2007). During non-breeding season, primarily winter, black-capped chickadees form multi-individual flocks (usually comprising 6 to 8 individuals, although flocks can range from as few as 2 individuals to as many as 12; Smith, 1991). Within these flocks, there is a strict linear dominance hierarchy: males tend to dominate females, with individual status (or rank) in the dominance hierarchy being primarily determined by sex and age (reviewed in Ratcliffe et al., 2007).

Diet during breeding season consists principally of animal material (insects, caterpillars, spiders; ca. 80-90% of diet; Smith, 1991). In non-breeding season, less animal material is consumed (reduced to ca. 40-50%), replaced instead by plant-based materials, primarily seeds (Smith, 1991). Black-capped chickadees, like other parids (family Paridae), store (or cache) their food (usually individual seeds, but also animal material; e.g. Heinrich and Collins, 1983). Individual caches are generally located in tree bark, broken branches, clusters of coniferous tree needles, under dry leaves, and in the snow (Sherry, 1984).

The frequency of food storing behaviour in chickadees and other parids, while occurring throughout the year, peaks in the fall months ahead of the winter season when conditions are harsh and other food sources are less abundant (Odum, 1942; Brodin, 2005; Pravosudov, 2006). Food caches are generally retrieved within days of storing (Sherry, 1984), however some caches are stored for a longer-term use. Experimental evidence shows chickadees can remember the location of food stores for at least 28 days

after caching (Hitchcock and Sherry, 1990), with some field studies suggesting retrieval can occur up to 40 days post-cache (Brodin and Ekman, 1994).

While black-capped chickadees have been studied in the context of their vocal behaviour (reviewed in Sturdy et al., 2007), this species (and Paridae generally) has been studied much more often in the context of their food-storing behaviour, and in particular, how this food-storing behaviour is supported by specialized cognitive processes including learning and memory (described below; reviewed in Clayton and Krebs, 1995; Krebs et al., 1996; Healy and Krebs, 1996; Clayton, 1998; Shettleworth, 2003; Healy and Hurly, 2004).

1.6. THE AVIAN HIPPOCAMPUS AND SPATIAL COGNITION

As described above, food-storing birds including parids, as well as corvids (family Corvidae), demonstrate a remarkable capacity for spatial memory (reviewed in Kamil and Balda, 1990; Clayton and Krebs, 1995; Shettleworth, 2003). However, food-storing birds are not the only taxa who display enhanced capabilities for spatial behaviour and memory. For example, homing pigeons, a domesticated breed of rock dove (*Columba livia*) have been used since ancient times as message carriers given their acute spatial and navigational abilities (reviewed in Wiltschko and Wiltschko, 2003; Mehlhorn and Rehkämper, 2009). Likewise, obligate brood parasites, including brown-headed cowbirds (*Molothrus ater*), lay their eggs in the nests of other species, and rely on spatial memory when searching for, and later returning to, nests to parasitize (reviewed in Guigueno and Sherry, 2017; Sherry and Guigueno, 2019). Together, these avian taxa (as well as others displaying prominent spatial capabilities) are sometimes referred to collectively as "memory specialists" (e.g., Basil et al., 1996; Payne et al., 2021) or "spatial specialists" (e.g., Phillmore et al., 2022).

In all these above-mentioned cases, spatial behaviour (food-storing, navigation, and brood parasitism) is supported by the same specialized neural structure: the hippocampus (reviewed in Krebs et al., 1989; Sherry et al., 1989; Lee et al., 1998; Bingman et al., 2003; Sherry and MacDougall-Shackleton, 2015; Guigueno and Sherry, 2017). Evidence for the role of the hippocampus in spatial behaviour and memory largely stem from lesion studies. For example, homing pigeons with bilateral hippocampal

lesions have difficulties returning to home roosts (Bingman et al., 1984; Bingman and Mench, 1990). In black-capped chickadees, bilateral hippocampal lesions prevent birds from retrieving previously stored seeds at levels greater than chance (Sherry and Vaccarino, 1989). Further, chemical or physical lesions of the chickadee hippocampus can also impair performance on a wide range of spatial memory tasks (Hampton and Shettleworth, 1996; Shiflett et al., 2003; Shiflett et al., 2004), and systemic suppression of hippocampal neurogenesis in black-capped chickadees using the toxin methylazoxymethanol impairs performance on spatial learning tasks (Hall et al., 2014b; Guitar and Sherry, 2018).

Despite the ca. 200-300 million years in which birds and mammals have evolved independently of one another (Hedges et al., 1996), both the avian and mammalian hippocampus are generally considered to be functionally homologous (reviewed in Lee et al., 1998; Colombo and Broadbent, 2000; Macphail, 2002). Both structures evolved from the ancient reptilian dorsomedial cortex (reviewed in Striedter, 2016) and are derived embryologically from the same telencephalic structures (e.g., Källen, 1962). Further, both structures share similar cell types, as well as some similar patterns of connectivity (e.g., Montagnese et al., 1996; Kempermann et al., 2004). Lastly, both structures show evidence of synaptic strengthening via long-term potentiation (Wieraszko and Ball, 1993; Yan-You Huang et al., 1996; Margrie et al., 1998), evidence of neurogenesis (reviewed in Kempermann, 2002; Sherry and Hoshooley, 2010) and, perhaps most importantly, evidence of experience-dependent neural plasticity (e.g., Kempermann et al., 1997; Clayton, 1998).

In birds, evidence of experience-dependent hippocampal plasticity is clearly observed when comparing spatial specialists to non-specialists: spatial specialists tend to have larger hippocampi, relative to the telencephalon, than non-spatial specialists; as seen when comparing homing to non-homing pigeons (e.g., Rehkämper et al., 1988), food-storing to non-food-storing birds (Krebs et al., 1989; Sherry et al., 1989), brood parasites to non-brood parasites (e.g., Reboreda et al., 1996), and migratory to non-migratory songbird subspecies (e.g., Pravosudov et al., 2006). Interestingly, changes in hippocampal plasticity are also observed within certain spatial specialist taxa, particularly

as the demands on ecologically relevant spatial memory change, for example over the course of the annual cycle (reviewed in Sherry and MacDougall-Shackleton, 2015).

Indeed, the bulk of the previous research investigating seasonal hippocampal plasticity in birds has focused primarily on food-storing birds, including the black-capped chickadee (reviewed in Sherry and Hoshoooley, 2009; Sherry and Hoshooley, 2010). As described above, food-storing behaviour in chickadees fluctuates seasonally, with more food-storing occurring in fall (e.g., Pravosudov, 2006), and while several studies have documented seasonal change in the hippocampus of food-storing birds (particularly volumetric changes and changes in neurogenesis; reviewed in Sherry and MacDougall-Shackleton, 2015), several important questions remain as to how the hippocampus varies with the demand on spatial cognition. Further, the presence of a seasonally fluctuating behaviour supported by a specialized brain region supporting this behaviour make food-storing birds, like black-capped chickadees, ideal models to study the mechanisms underlying the interaction between behavioural and neural plasticity.

CHAPTER 2: MANUSCRIPT

2.1 INTRODUCTION

Food-storing birds have long been used in both laboratory- and field-based research to study learning and memory (reviewed in Clayton and Krebs, 1995; Krebs et al., 1996; Shettleworth, 2003; Healy and Hurly, 2004). Specifically, chickadees (genus *Poecile*), including black-capped chickadees (*P. atricapillus*), have been studied extensively to examine the interaction between food-storing behaviour and the underlying brain regions supporting spatial memory, particularly the hippocampus (reviewed in Clayton, 1998; Sherry and Hoshooley, 2009; Sherry and Hoshooley, 2010; Sherry and MacDougall-Shackleton, 2015; Pravosudov et al., 2015). Chickadees intensely store (or cache) food during the late summer and fall in preparation for the food-scarce winter (Odum, 1942; Brodin, 2005; Pravosudov, 2006). Despite predictable seasonal changes in storing rates, how the chickadee hippocampus changes seasonally, particularly as the demands on spatial memory change over the year, varies across studies (reviewed in Sherry and Hoshooley, 2009; Sherry and Hoshooley, 2010; Sherry and MacDougall-Shackleton, 2015; Pravosudov, 2022).

In the first study to examine seasonal plasticity in the hippocampus of wild-captured chickadees (Barnea and Nottebohm, 1994), birds were captured throughout the year, injected with the cell-birth marker tritiated thymidine ([^{3}H]-TdR), released back into the wild, and recaptured six weeks later. Birds recaptured in October had a higher percentage of new neurons in the hippocampus compared to birds captured at other times over the year (Barnea and Nottebohm, 1994). Similarly, black-capped chickadees captured in October had a larger hippocampus by volume (even when controlling for factors such as overall brain size, sex, and age) compared to birds captured in the winter (December, February), spring (April), and summer (June, August; Smulders et al. 1995). This larger hippocampal volume observed in the birds captured in October is a result of more total cells (neuron and glia) in the hippocampus in October (Smulders et al., 2000). Taken together, these findings suggest the increased demand on hippocampus-dependent spatial memory in the fall, specifically while chickadees are acquiring new memories

during storing, is supported by a larger hippocampus with more neurons and increased rates of neurogenesis.

Subsequent studies of black-capped chickadees have, however, all failed to replicate the fall peak in hippocampal volume, neuron number, and neurogenesis initially described by Barnea and Nottebohm (1994) and Smulders et al. (1995; 2000). A notable exception is Lange et al. (2021), who replicated the fall peak in overall hippocampal volume and total hippocampal neuron number in a closely related Eurasian species of food-storing bird, the willow tit (*Poecile montanus*): willow tits captured in September had a larger hippocampus by volume, and more neurons in the hippocampus, compared to birds captured in July, August, November, or April, coinciding with the September peak in food-storing observed in this species (Lange et al., 2021). In contrast, Hoshooley and Sherry (2004) found no difference in any hippocampal attribute (volume, neuron number, neuron production, or apoptosis) among black-capped chickadees captured over the fall and winter months (each month from October to March). Later, Hoshooley and Sherry (2007) did detect a difference in the hippocampus when birds were combined into spring (captured from February 28-April 11) and fall (captured October 5-November 25) groups, however the effect was opposite that observed in the initial studies (by Barnea and Nottebohm, 1994; Smulders et al., 1995; 2000): chickadees captured in spring, not fall, had a larger hippocampus by volume, containing more neurons. And, while Hoshooley and Sherry (2007) did not detect a difference in the number or density of new neurons in the hippocampus between spring and fall birds, a parallel study by Hoshooley et al. (2007) showed that indeed, birds captured in January had significantly more new neurons (as measured by bromodeoxyuridine [BrdU], another exogenous cell-birth marker), and a significantly higher density of new neurons in the hippocampus compared to birds captured in October (i.e., in contrast to Barnea and Nottebohm, 1994) and July. These findings suggest that it is not the increased demand on spatial memory during storing in the fall, but rather increased demand while birds are actively recalling the location of their stores in winter, that necessitates a larger hippocampus containing more neurons and increased rates of neuron proliferation.

A variety of other behavioural, physiological, and neuroanatomical changes in songbirds, particularly those related to vocal behaviour and birdsong, can be reliably

induced in both the laboratory and the field by changes in, and manipulation of, photoperiod (or day length; reviewed in Brenowitz, 2004). However, it remains unclear how (or even whether) photoperiod regulates the seasonal plasticity in the hippocampus in food-storing birds such as black-capped chickadees (reviewed in Phillmore and MacDougall-Shackleton, 2007; Sherry and Hoshooley, 2010; Sherry and MacDougall-Shackleton, 2015; LaDage, 2022). Experimental manipulations of photoperiod, while sufficient to induce some changes in food-storing behaviour (particularly those observed in fall) in black-capped chickadees housed in the laboratory (Shettleworth et al., 1995; Krebs et al., 1995), do not produce the expected neuroanatomical changes in the hippocampus observed in seasonal studies of free-ranging chickadees (e.g., volume, Krebs et al., 1995; MacDougall-Shackleton et al., 2003; neurogenesis, Hoshooley et al., 2005).

It is possible captivity plays a role in the variability of these findings (Sherry and MacDougall-Shackleton, 2015; Pravosudov et al., 2015; Phillmore et al., 2022; Pravosudov, 2022). Smulders et al. (1995; 2000) and Lange et al. (2021) sacrificed birds on the same day of capture. In contrast, Hoshooley and Sherry (2004) individually housed birds indoors, with free access to food, for 1-2 weeks post-capture, while Hoshooley et al. (2007) and Hoshooley and Sherry (2007) housed birds outdoors in aviaries for 1 week and 6 weeks, respectively. The use of BrdU as a cell birth marker in these studies required that birds be housed in captivity for some portion of the experimental timeline, as it generally requires time such that BrdU can incorporate into newly divided cells (Balthazart and Ball, 2014b), and for newly-divided cells to be recruited to the hippocampus, which is estimated to occur on the timeframe of ca. 1 week (Barnea and Pravosudov, 2011). However, keeping birds in captivity introduced a confound to these studies that may have affected the results: bringing birds into captivity reduces the size of the hippocampus in food-storing and non-food-storing birds and is known to alter hippocampal neurogenesis (reviewed in Phillmore et al., 2022).

Captivity may also be why studies have not detected a direct effect of photoperiod on the hippocampus, despite consistently showing an effect on nuclei of the vocal control system. While standard laboratory housing (either in cages or aviaries) generally does not prevent a bird from vocalizing, it can place restrictions on the ability of a food-storing

bird to perform natural, ecologically relevant spatial behaviours such as food storing and retrieval. There is evidence from mountain chickadees (*Poecile gambeli*; e.g., LaDage et al., 2009; LaDage et al., 2010) suggesting that giving birds the opportunity to store food while housed in captivity might mitigate some of the negative effects of captivity on hippocampal neural plasticity.

However, food-storing experience in captivity is not comparable, both in terms of caching frequency, and total space/locations available for caching, to that which occurs in the wild. For example, MacDougall-Shackleton et al. (2003) detected no effect of photoperiod of hippocampal volume in black-capped chickadees; in this study birds were only provided with one opportunity every 4 days (11 × 30-minute sessions total over 44-day study) to store food in captivity. Across all photoperiodic conditions (even those mimicking fall and winter), the mean number of stored seeds never exceeded 4 seeds cached per 30-minute session. This is in stark contrast to field studies showing that during the fall "peak" in caching, chickadees and tits can store from 60 to over 100 seeds per hour (Brodin, 2005; Lange et al., 2021). Further, Hoshooley et al. (2005) found no effect of photoperiod on hippocampal neurogenesis in black-capped chickadees. These birds were not provided with any sessions to cache food for the duration of the study (29 days); instead, birds were housed individually in acoustic isolation chambers with *ad libitum* access to food). This particular form of captivity, especially when compared to a food-storing bird's natural experiences in the wild, could be considered a form of impoverished environment, known to downregulate hippocampal neurogenesis in birds, as in rodents (Renner and Rosenzweig, 1987; Barnea and Pravosudov, 2011). Given that captivity drastically diminishes several aspects of hippocampal neural plasticity, the lack of food-caching opportunities available to chickadees in previous laboratory-based studies (e.g., MacDougall-Shackleton et al., 2003; Hoshooley et al., 2005) might have compromised the ability to detect a main effect of photoperiod.

Captivity-induced stress may also contribute to differences in hippocampal plasticity across studies (reviewed in Sherry and MacDougall-Shackleton, 2015; Pravosudov et al., 2015; Phillmore et al., 2022), as the hippocampus plays a key role in mediating some of the physiological responses to stressors via activation of the hypothalamic-pituitary-adrenal (HPA) axis (reviewed in Smulders, 2017). Placing a wild

bird into laboratory captivity leads to an increase in plasma glucocorticoids (e.g., Marra et al., 1994; Dufty and Belthoff, 1997), and, as previously described, captivity negatively affects hippocampal volume and neurogenesis in birds. While it is difficult to determine the specific role stress plays relative to the other factors associated with captivity (i.e., reduced space, reduced memory demand; LaDage, 2015), it appears as though it may be stress associated with transferring a bird from the wild into captivity that leads to changes in the hippocampus (reviewed in Sherry and MacDougall-Shackleton, 2015; Pravosudov et al., 2015; Phillmore et al. 2022), as mountain chickadees hand-reared in captivity from 10 days post-hatch showed no difference in hippocampal neurogenesis and neuron number as their wild counterparts (Roth et al., 2012).

Therefore, the objective of this study was to examine seasonal patterns of hippocampal plasticity in a large sample (N=48) of wild black-capped chickadees not exposed to any extended period of laboratory captivity. I captured black-capped chickadees from the wild while in one of the three principal photoperiodic conditions: photostimulated (day length increasing), photorefractory (consistent long days), and photosensitive (short days). I estimated photoperiodic condition *a priori* based on day length and assessed photoperiodic condition post-mortem by examining gonads. To minimize effects of captivity, birds were sacrificed shortly (ca. 1 hour) after capture in the field and were not introduced to any of the constraints associated with laboratory captivity.

I also aimed to clarify whether seasonal patterns of neural plasticity varied between male and female chickadees: while Barnea and Nottebohm (1994) and Smulders et al. (1995) included sex as a factor in their design (and found no main effect), Hoshooley et al. (2007) and Hoshooley and Sherry (2007) only used male chickadees as subjects, therefore it is unclear whether the same seasonal patterns observed in these studies also applied to female chickadees.

I quantified two measures of neural plasticity: volume (using cresyl violet) and neurogenesis (using doublecortin immunohistochemistry). While prior seasonal studies have quantified neurogenesis using exogenous markers (e.g., [^{3}H]-TdR, Barnea and Nottebohm, 1994; BrdU, Hoshooley et al., 2007), to my knowledge, no study has

examined seasonal variation in neurogenesis in black-capped chickadees using this endogenous marker.

Overall, I predicted that birds captured in winter, while photosensitive, would show a larger hippocampus by volume; likewise, I predicted that photosensitive birds would have more new neurons in the hippocampus. I also hypothesized these differences would not vary between male and females (as in Petersen and Sherry, 1996; Pravosudov and Clayton, 2002; Roth et al., 2011).

2.2 MATERIALS AND METHODS

All procedures were approved by the Canadian Wildlife Service (permit no. SC4055) and by the Dalhousie University Committee on Laboratory Animals (permit no. 21-021), in accordance with the guidelines of the Canadian Council on Animal Care.

2.2.1 SUBJECTS AND FIELD SITES

I captured black-capped chickadees (*N*=48; 26 males, 22 females) from the wild from ten different private sites (with landowner permission) in Halifax, Nova Scotia (Figure 1). The mean distance from capture sites to the laboratory (direct distance) was 14.5 km (range = 1.9 – 23.3 km). To avoid targeting entire micropopulations and/or entire flocks of chickadees, I attempted to minimize the number of birds captured from one specific site per season, as well as from one specific site over the entire year. The maximum number of individuals collected from one location in one photoperiodic condition (i.e., over one season, out of 16) was 4, and the maximum number of individuals collected from one location overall (i.e., over the entire year, out of 48) was 10.

2.2.2 CAPTURE SEASONS

I made *a priori* estimations of photoperiodic condition primarily based on local day length; I also considered the timelines previously used in seasonal studies of black-capped chickadees in the area (e.g., Phillmore et al., 2011, Phillmore et al., 2015). The capture windows were additionally informed by specific dates authorized under the capture permit granted by the Canadian Wildlife Service. Ultimately, I captured photostimulated birds (*n*=16) from 25 March – 10 April 2021, photorefractory birds (*n*=16) from 13 August – 15 September 2021, and photosensitive birds between 1 December 2021 – 26 January 2022, in two groups: one group (*n*=9) between 1-18 December 2021 and another (*n*=7) between 17-26 January 2022. Because of the protracted capture period in the photosensitive group (56 days, with a 30 day gap between birds captured in December and January) compared to the photostimulated (16 days) and photorefractory (33 days) groups, and that birds captured in December were experiencing decreasing day length (pre-Winter solstice) whereas those captured in

January were experiencing increasing day length (post-Winter solstice), I consider these two groups separately in my analyses (December birds denoted as 'Photosensitive A'; January birds denoted as 'Photosensitive B'), unless otherwise specified. I calculated sunrise time and total day length using the online sunrise/sunset calculator available from the Natural Research Council (https://nrc.canada.ca/en/research-development/products-services/software-applications/sun-calculator/) and the approximate coordinates of each capture site. Table 1 shows the mean and range of day lengths for birds captured across all four seasons; these data are also plotted in Figure 2. Photoperiodic condition was later assessed by a post-mortem examination of gonads (described below).

2.2.3 CAPTURE PROCEDURE

All birds were captured outdoors using walk-in (Potter) traps baited with black oil sunflower seeds. Traps were placed on a wooden platform raised ca. 1m off the ground. I used audio recordings of black-capped chickadee mobbing calls to attract birds to the traps; recordings were played from a waterproof portable wireless speaker placed near the raised platform. Once a bird entered the trap, I stopped audio playback and immediately extracted the bird from the trap. The bird was then aged and sexed (if necessary; procedure described below) and bled within 3 minutes of capture. I collected blood to examine seasonal variation in hematocrit (described below) as well as for future analyses of seasonal variation in glucocorticoid (e.g., corticosterone, CORT) and reproductive hormones (e.g., LH, T, and E2). Collecting blood within 3 minutes is standard practice when quantifying baseline CORT in birds, including in studies of chickadees (e.g., Pravosudov et al., 2002; Burns and Bonier, 2020), as it is generally accepted that blood collected after 3 minutes may contain elevated levels of CORT that reflect the acute stress response associated with capture and/or blood collection, rather than basal levels (Romero and Romero, 2002; Romero and Reed, 2005). Further, all birds were captured between the hours of 08:30 and 11:30, at least 60 minutes after the local sunrise time (mean = 146 minutes, range = 77 – 284 minutes) to avoid the influence of diel variation in circulating hormones (as in Burns and Bonier, 2020).

I did not use song playback to lure chickadees to the traps (as is frequently used in similar studies; e.g., Phillmore et al., 2011; Lange et al., 2021) because of the potentially

confounding effects of song playback on baseline levels of CORT and T in songbirds (e.g., Pinxten et al., 2004; Landys et al., 2007; but see Fokidis et al., 2011; Rosvall et al., 2012). I specifically opted for mobbing calls as Burns and Bonier (2020) found no difference in baseline plasma CORT between black-capped chickadees captured (in mist-nets) with mobbing call playback and birds captured (in walk-in traps) without any auditory playback, suggesting that call playback itself does not cause the same surge in CORT that is found when using song playback. I used four different recordings of chickadee mobbing calls, all used with permission from the MacAulay Library at The Cornell Lab of Ornithology (recording IDs: ML14646, ML205639, ML195523) and xeno-canto (recording ID: XC544961).

To collect blood, I made a small puncture using a 26-gauge needle tip in the left brachial vein, and collected whole blood using 70 μL heparinized micro-hematocrit capillary tubes (Fisher). I collected a maximum of 2 full tubes (ca. 120–140 μL) per bird, stopping all blood collection within 3 minutes of the bird entering the trap. If I collected 2 full tubes prior to 3 minutes elapsing, or if a hematoma occurred, I stopped collecting blood. Microcapillary tubes were then sealed with Critoseal (McCormick Scientific) and stored on ice (in 15 mL Falcon tubes) until processing. After collecting blood, I immediately applied pressure to the puncture site with a cotton ball until bleeding stopped, after which I collected standard morphological measurements. I measured wing chord and tail length (central rectrix method; Pyle et al., 1997) to the nearest 1 mm using a bird banding ruler, and quantified amount of body fat (as observed in the furculum) using a standard 7-point scale (e.g., Dunn, 2003; Bird Studies Canada, 2019). After all measurements were collected, birds were placed into a cloth bird bag.

While I was mindful of minimizing the amount of time between capture and sacrifice, I generally did not leave field sites immediately after capturing one lone bird; once the first captured bird was secured in a bird bag, I usually reset traps and began playback again in an attempt to capture additional birds. If I did not capture another bird within ca. 20-30 minutes of capturing the first bird, I left the field site for the laboratory. All birds were transported to the laboratory (by vehicle) within 40 minutes of capture (average = 21 minutes, range = 7 – 39 minutes).

2.2.4 SEXING AND AGING PROCEDURE

I aimed to have approximately equal numbers of males and females in each condition; therefore there were instances where I measured wing chord and tail length prior to blood collection. I compared wing chord and tail length to previous morphological data compiled from chickadees captured during previous fieldwork; I released any birds that did not fit the intended sex quota per condition. However, given the degree of overlap in wing chord and tail length between male and female black-capped chickadees (Pyle et al., 1997), sample sizes across conditions are not exactly equal (photosensitive: 8M, 8F; photorefractory: 10M, 6F; photosensitive: 8M, 8F). The variation in wing chord, tail length, and tarsus length (which I measured post-perfusion; described below), separated by sex, for birds captured in this study are presented in Figure 3.

Because black-capped chickadees are sexually monomorphic, they are generally difficult to age in the field (Pyle et al., 1997; Fylling et al., 2018), particularly when there are time constraints associated with capturing and handling (i.e., ensuring a blood draw within 3 minutes). I was primarily interested in capturing adult birds for this study. I used the calendar-based aging system (described in Pyle et al., 1997) and considered all after-hatch-year birds (AHY; i.e., a bird at least in its second calendar year) birds as "adults". I did not age birds in the field during the first (photostimulated) season as I assumed they were all AHY, given that capture occurred prior to egg-laying in the capture area (loosely based on egg dates described by Foote et al., 2020). I aged birds in the remaining seasons (photorefractory/photosensitive) in the field using a combination of age-distinguishing field marks described by Pyle et al. (1997), including the wear, shape, and color of the outer rectrices, the texture of undertail coverts, and the color of the upper mandible lining. Age could only be confirmed during a post-perfusion examination of skull pneumatization. Birds showing any evidence of field marks associated with juvenile age (i.e., hatch-year, HY) such as pointed outer rectrices and/or no white along the edge of the inner web of the rectrix, were released and not bled.

However, while capturing 11 birds in the photorefractory group (August-September), and one in the photosensitive A group (December) there was a discrepancy between age determined in the field and the age determined after examining skull

pneumatization. In all cases, a bird displaying all the purported field marks of an adult was determined to be a juvenile after examining the skull for pneumatization. In these cases, I deferred to the age as determined by skull pneumatization, as this is a more robust and reliable indicator of age in songbirds (Pyle et al. 1997), particularly in black-capped chickadees (Yunick, 1980; Yunick, 1981; Fylling et al., 2018). Consequently, the photorefractory group comprised both juvenile and adult birds (11 juveniles, 5 adults), whereas the photostimulated and photosensitive groups consisted primarily of adult birds only (photostimulated: 0 juveniles, photosensitive A: 1 juvenile; photosensitive B: 0 juveniles). I therefore excluded all juvenile birds from subsequent analyses. Table 2A shows a breakdown of all subjects captured, separated by age and sex. Table 2B shows a breakdown of all subjects included in analyses, separated by sex.

2.2.5 EUTHANASIA AND PERFUSION

Upon arrival at the laboratory, birds were removed from the cloth bird bag and euthanized with a 0.1 cc intraperitoneal injection of euthanyl. The mean time between capture and euthanasia was 64 minutes (range = 31 – 94 minutes). In instances where more than one bird was brought to the laboratory at the same time, birds were euthanized in the order of capture. After euthanasia was confirmed (via toe pinch), I weighed the bird using an analytical balance (Sartorius TE214S), confirmed any outstanding morphological measurements not completed in the field, and collected 5-6 primary feathers from the bird's left wing; feathers were placed in a paper coin envelope, sealed, and stored in a dark and dry area (for future analyses).

Next, I made an incision in the neck area and severed the jugular vein, collecting trunk blood into heparinized microcapillary tubes, which were then sealed and stored on ice until processing. Birds were then transcardially perfused with heparinized phosphate-buffered saline (PBS; pH=7.4), then with 4% paraformaldehyde (PFA). Brains were extracted from the skull, weighed on an analytical balance (for post-perfusion weight, to the nearest 0.0001 g) and soaked in 4% PFA for 24-72 hours until uniformly fixed (confirmed by visual inspection). Brains were removed from PFA, weighed again (for post-fix weight), and transferred into a 30% sucrose (in PBS) solution for cryoprotection until saturated. I then weighed the samples once more (for post-cryoprotection weight)

prior to flash freezing on pulverized dry ice; brains were stored at -80ºC in an aluminum foil wrapper until processing. Brains collected from two birds required additional processing due to insufficient clearing and fixation during perfusion (e.g., entire heads were soaked in PFA for >72 hours until sufficiently fixed); their post-perfusion, post-fix, and post-cryoprotectant weights are consequently excluded from analyses.

Following brain extraction, I assessed photoperiodic condition and sex via examination of the gonads. In females, I visually assessed the stage of ovary development using the ordinal scale developed for black-capped chickadees by MacDougall-Shackleton et al. (2003), described in Table 3. In males, the left testis was removed and the length and width were measured (to the nearest 0.1 mm) using dial calipers. These measurements were used to calculate testis volume using the formula of an ellipsoid:

$$\frac{4}{3}\pi a^2 b$$

where *a*=width/2; *b*=length/2. Male black-capped chickadees are considered to be in breeding condition (i.e., photostimulated) when testis volume is greater than 20 mm^3 (Phillmore et al., 2006).

Later, once all birds were collected for this study, I also performed post-perfusion measures of each bird's left tarsus (measuring length to the nearest 0.1 mm) using dial calipers. To ensure consistency in measurements, I measured tarsus twice and report the average of the two measurements (see Figure 3C). The mean coefficient of variation between the two tarsus measurements was 0.93%.

2.2.6 BLOOD PROCESSING

All microcapillary tubes containing blood collected from the field, as well as a portion of tubes containing trunk blood, were processed on the day of capture, as soon as possible after perfusions were completed. All tubes were centrifuged at most within 4 hours of collection; vertebrate blood can be stored at cool temperatures for much longer without compromising hormonal integrity or stability (e.g., Taylor and Schuett, 2004; Khonmee et al., 2019, Burns and Bonier, 2020). Tubes were spun on a microhematocrit centrifuge (Unico C-MH30) at 12,000 rpm for 10 minutes. After centrifugation, I

measured tubes for hematocrit using a capillary microhematocrit reader (Unico C-MH30). Hematocrit fraction was calculated by dividing packed blood cell measurement by the total blood measurement (packed cells + plasma). While there is some evidence that hematocrit may vary with season, sex, and reproductive condition in some species (reviewed in Fair et al., 2007), to my knowledge no study has examined seasonal variation in hematocrit in black-capped chickadees.

Following hematocrit measurement, plasma was separated from the blood pellet using a 50 µL syringe (Hamilton) and aliquoted into a microcentrifuge tube. Next, I used a separate syringe plunger to remove the remaining red blood cell pellet from the microcapillary tube, storing it in a separate microcentrifuge tube. All tubes were then briefly pulsed on a tabletop centrifuge (Costar Mini) before being stored at -20ºC. Some microcapillary tubes from birds captured during the photorefractory season could not be centrifuged due to mechanical issues with the microhematocrit centrifuge; therefore not measure hematocrit from these samples. In these cases, whole blood was aliquoted into microcentrifuge tubes and centrifuged on a tabletop centrifuge at 10,000 rpm at for 15 minutes. As before, plasma was separated from the blood pellet using a syringe after centrifugation, and plasma and red blood cell pellet were stored in separate microcentrifuge tubes.

I opted to not centrifuge all trunk blood that was collected, instead keeping a portion of the trunk blood as whole blood. In these cases, whole blood was aliquoted into microcentrifuge tubes and stored at -20ºC. To avoid cross-contamination, I used a separate syringe to aliquot plasma and whole blood, sanitizing the syringe using distilled (Type II) water and 90% ethanol between subjects, as well as between field and trunk samples.

2.2.7 TISSUE SECTIONING

At the time of sectioning, each brain was removed from the -80ºC freezer (in order of capture) and placed in a cryostat (Leica CM1950); once warmed to ca. -16ºC, I separated the brain midsagittally (i.e., into left and right hemispheres) using a razor blade. One hemisphere was then returned to the -80ºC (for future processing) and the other was mounted on the cryostat and sectioned coronally at 30 µm into four sets. I alternated

which hemisphere was sectioned and which was returned to the freezer based on order of capture (as in Lange et al., 2021); the hemisphere to be sectioned from the first subject was chosen at random. I therefore had approximately equal numbers of left and right hemispheres in each condition. Once sectioned, tissue was stored in cryopreservative (30% sucrose, 30% ethylene glycol, 1% polyvinylpyrrolidone in PBS) in sterile 24-well trays at -20ºC until processing. At the time of sectioning, birds (and trays) were assigned a random three-digit identification number such that all subsequent tissue processing, microscopy, and cytometry could be completed blind to condition, sex, and age.

2.2.8 BRAIN REGION MORPHOMETRY

2.2.8.1 HISTOLOGY

One set of tissue was removed from the freezer, washed once in PBS (to remove cryopreservative), float mounted serially on 1% gelatinized slides (Ultri-Dent), then allowed to air dry for 48 hours. I then stained tissue for Nissl substance with cresyl violet, following a protocol described by Ormerod et al. (2003) adapted specifically for avian brain tissue. Slides were rinsed briefly in distilled water to remove salts, then placed in a 2% cresyl violet acetate (Acros Organics) in distilled water solution for 5-7 minutes. Slides were again rinsed in distilled water and then submerged in glacial acetic acid in 70% ethanol for 30 seconds, before being serially dehydrated in 95% (two rinses) and 100% ethanol for 2 minutes each. Slides were then cleared with Neo-Clear (Harleco) for 10 minutes, and then coverslipped using Permount (Fisher).

2.2.8.2 MICROSCOPY AND VOLUME RECONSTRUCTION

Cresyl violet-stained slides were imaged under brightfield illumination using an Olympus DP80 camera paired to an Olympus BX-51 microscope with an automated motorized stage (Prior ProScan II). I used the Multiple Image Alignment feature in Olympus cellSens Dimension (version 1.14) to scan and capture individual images (captured using a 4× objective; 680×512 resolution), stitching them into whole slide images (see Figure 4A for example). Whole-slide images were then imported into the FIJI version of ImageJ (version 1.53q), where I used the Polygon function to trace the

area of both the hippocampus (Figure 4B) and the telencephalon (Figure 4C). I traced the area of the hippocampus in every section it was visible (120 µm interval), following the boundaries described by Sherry et al. (1989). I then calculated the volume (*V*) between each section using the formula for a frustrum (truncated cone):

$$V = \frac{W}{3(A_1 + (\sqrt{A_1} \times \sqrt{A_2}) + A_2}$$

where A_1 and A_2 are areas from successive sections and *W* is the interval between the sections. I was then able to calculate total hippocampal volume by summing the volumes of each frustrum. I used the same method to calculate telencephalon volume, except I sampled from every second mounted section (240 µm interval). This sampling method has been used extensively to reconstruct the volume of both the hippocampus and the telencephalon of black-capped chickadees (e.g., Hoshooley et al., 2007; Phillmore et al., 2015) and other songbirds (e.g., European starlings, *Sturnus vulgaris;* Hall and MacDougall-Shackleton, 2012; brown-headed cowbirds, Guigueno et al., 2016), as well as other vertebrates (e.g., Long-Evans rats; Galea et al., 2000). When measuring both hippocampal and telencephalon volume, I was able to account for any sections that were damaged, missing, or otherwise unable to be traced by adjusting the sampling interval (*W*) accordingly.

2.2.9 DOUBLECORTIN (DCX) IMMUNOHISTOCHEMISTRY

I quantified neurogenesis using standard immunohistochemical methods to stain for the microtubule-associated protein doublecortin (DCX), a well-used endogenous marker of adult neurogenesis in birds and other models (e.g., Brown et al., 2003; Couillard-Despres et al., 2005; Balthazart et al., 2008). In birds, DCX is a valid and reliable marker of immature neurons (Balthazart and Ball, 2014a; Balthazart and Ball, 2015b) and has been used extensively to quantify avian neurogenesis, including in previous studies investigating hippocampal neural plasticity in chickadees (e.g., Fox et al., 2010; LaDage et al., 2010; Chancellor et al., 2011; Freas et al., 2012). The specific DCX immunohistochemistry protocol used in this study was adapted principally from the

procedure described by Vandries et al. (2019), with minor modifications based on the protocols of Aronowitz et al. (2021) and Phillmore et al. (2022), particularly to optimize its use with chickadee tissue. Brains were processed in six batches; I used block randomization to ensure each batch comprised an equal sample of brains from each capture season and sex.

2.2.9.1 DCX IMMUNOHISTOCHEMISTRY PROTOCOL

Trays were removed from the -20ºC freezer and left overnight in a 4ºC refrigerator. The following morning, sections were transferred into clean trays containing Tris-buffered saline (TBS, pH=7.6). Sections were washed three times with TBS and then placed into 3% hydrogen peroxide in TBS for 15 minutes. I then washed sections four more times in TBS before incubating in a blocking buffer consisting of 0.2% bovine serum albumin (Vector, SP-5050) and 5% normal goat serum (Vector, S-1000) in 0.1% Triton-X in TBS (TBS-T) for 30 minutes. Sections were then incubated in a 1:2000 dilution of the primary polyclonal antibody rabbit anti-doublecortin IgG (Abcam, ab18723, RRID:AB_732011) in blocking buffer for ca. 48 hours (with agitation). While this particular DCX antibody has been previously validated for use in other songbird species (e.g., canaries, Vandries et al., 2019; zebra finches, *Taeniopygia guttata*, Aronowitz et al., 2021) it has not, to my knowledge, been used to quantify doublecortin immunoreactivity in black-capped chickadees. To therefore confirm the specificity of the antibody with chickadee tissue, I omitted primary antibody from one well in all trays in one batch (n=6). Negative control wells either contained blocking buffer without antibody or TBS-T only; in both cases, tissue incubated in wells without primary antibody produced no immunostaining.

After 48 hours, sections were washed three times with TBS, then incubated for 2 hours (at room temperature, with agitation) in the biotinylated secondary antibody goat anti-rabbit IgG(H+L) (Vector, BA-1000, RRID:AB_2313606) diluted at 1:500. After three more washes, sections were incubated for 1 hour in an avidin-biotin horseradish peroxidase complex (VECTASTAIN Elite ABC-HRP Kit, Vector, PK-6100, RRID:AB_2336819) diluted at 1:400. Tissue was washed twice more prior to being reacted with a 0.04% 3,3'-diaminobenzidine (DAB) solution activated with urea-

hydrogen peroxide (SIGMAFAST DAB, Sigma, cat nos. D4418/D4293) for ca. 2.5-3 minutes to visualize the antibody-avidin-biotin complexes. Sections were then washed four more times in TBS and stored (in TBS) at 4ºC prior until mounting. I then float-mounted DCX+ tissue serially on gelatinized slides and allowed sections to dry for 24-48 hours prior to dehydration with graded concentrations of ethanol, clearing with Neo-Clear, and coverslipping with Permount.

2.2.10 QUANTIFICATION OF DCX-IMMUNOREACTIVITY

I imaged DCX+ slides using an Olympus DP80 camera paired to Olympus BX-51 microscope with an automated motorized stage. I examined DCX-immunoreactivity (DCX-ir) using two approaches, quantifying: (i) the proportion of overall DCX-ir (cell bodies, neurites, axons) per microscope field of view (%DCX+ coverage); and (ii) the number of DCX+ cells per microscope field of view (FOV). I therefore had a more traditional cell counting technique, as well as a more objective measure of overall immunoreactivity, which, for example, counted neurites which may be stained, but may not have a cell body within the FOV.

2.2.10.1 SAMPLING STRATEGY

I sampled along the rostral-caudal (i.e., longitudinal) extent of the hippocampus, as in Guigueno et al. (2016) and Lynch (2018). I chose this sampling strategy as patterns of neurogenesis, including neurogenesis quantified using DCX-ir, can vary along the rostro-caudal axis of the avian hippocampus (e.g., Barnea and Nottebohm, 1994; Guigueno et al. 2016; Lynch, 2018; Mehlhorn et al., 2022). I sampled from 15 tissue sections per bird: 5 successive sections (interval = 120 μm) in the rostral hippocampus, 5 successive sections in the caudal hippocampus, as well as 5 successive sections between the rostral and caudal divisions (hereafter referred to as the "mid" division of the hippocampus).

I determined the rostral, mid, and caudal divisions in each bird by first imaging the hippocampus using a 4× objective, placing two rectangular sampling frames measuring 440 × 330 μm (area = 0.145 mm^2) within the hippocampus. The rostral division comprised the first five successive sections where two sampling frames could be

placed, without overlap, completely within the bounds of the hippocampus. Across all birds sampled, the first section of the rostral division was located 360-1680 μm (median = 960 μm or 8 sections) from the extreme rostral bound of the hippocampus defined by Sherry et al. (1989). Conversely, the caudal division comprised the last five successive sections where two sampling frames could be placed within the bounds of the hippocampus. Across all birds sampled, the last section of the caudal division was located 0-1680 μm (median = 360 μm or 3 sections) from the extreme caudal bound of the hippocampus. The mid division of the hippocampus was defined as the middle five successive sections between the above-mentioned rostral and caudal divisions.

2.2.10.2 REGIONS OF INTEREST

Within each tissue section, I sampled from four regions of interest (ROIs): two ROIs were located in the hippocampus (HP1 and HP2) and two were located in control regions outside the hippocampus (HA and LNP). Figure 5 shows a line drawing of the four ROIs in a representative section in each of the three divisions (rostral, mid, caudal) along the rostral-caudal axis. Some tissue was damaged, missing, or otherwise unable to be imaged; in such cases I was unable to capture all 60 images (4 ROIs per section × 5 sections per division × 3 divisions) per subject. The HP1 sampling frame was placed in the dorsolateral portion of the hippocampus, close to the dorsal surface of the brain, near the border between the hippocampus and the adjacent structure, hyperpallium apicale (HA). The HP2 sampling frame was placed in the ventromedial portion of the hippocampus, close to the midline, without overlapping with the subventricular zone. I opted to sample from these two divisions within the hippocampus as DCX-ir is also known to vary along the dorsal-ventral axis of the hippocampus (e.g., Phillmore et al., 2022).

2.2.10.3 CONTROL REGIONS

The sampling frame for HA was placed on the opposite side of the hippocampus-HA border (generally mirroring HP1), centered between the subventricular zone and the dorsal surface of the brain. HA has long been considered a standard control/comparator region in immunohistochemistry-based studies of seasonal hippocampal neurogenesis in

songbirds (e.g., Barnea and Nottebohm, 1994; Hoshooley et al., 2005; Hoshooley et al., 2007; Guigueno et al., 2016, Lynch, 2018; Phillmore et al., 2022), given its close proximity to the hippocampus in coronal sections. HA is generally implicated in upper-order sensory processing in birds (e.g., Bradley and Horn, 1979); some have considered it analogous to the secondary (or supragranular) layers of the primary visual cortex (Shimizu and Bowers, 1999).

However, a recent electrophysiology-based study found evidence for mammalian-like place cells not only in the hippocampus, but also in HA, in barn owls (*Tyto alba*; Agarwal et al., 2021), suggesting some degree of spatial coding may potentially occur in this region in birds. Therefore, I also opted to sample from the lateral nidopallium (LNP) as a non-spatial control region. The LNP sampling frame was located in the most lateral portion of the nidopallium, close to the lateral edge of the brain (Figure 5). I chose LNP as a separate negative control region, in large part due to its distance from the hippocampus. The nidopallium comprises a wide portion of the avian telencephalon (Iwaniuk and Hurd, 2005); the functionality of its lateral subdivision is relatively unclear but appears to play some role in social behaviour in birds, including sexual imprinting (Bischof and Hermann, 1998; Sadamanda and Bischof, 2006).

2.2.10.4 IMAGE ANALYSIS AND CYTOMETRY

I captured images of each sampling using a 20× objective. The dimensions of the captured images were the same as the sampling frames (440 × 330 μm, area = 0.145 mm^2). All images were imported into the FIJI version of ImageJ for analysis. I calculated the proportion of immunoreactivity in each image collected (n=2504 images total, n=1868 excluding data from juvenile birds) based on a modified protocol described originally by Diez et al. (2021). I first removed background staining from images using the Threshold_Colour automated color thresholding plug-in; I specifically used the IJ_IsoData thresholding algorithm (ImageJ's original implementation of the IsoData algorithm described by Ridler and Calvard, 1978; see example in Figure 6D); this specific algorithm has been used previously to threshold images of immunolabelled cells in brain sections (e.g., Karunasinghe et al., 2018; Venkatesh et al., 2019; Takesono et al., 2022). Images were then converted to greyscale (8-bit), where I used the Analyze

Particles tool to calculate the proportion of immunoreactivity in each image. I accounted for any potential artifacts in the images (e.g., dust particles, dried mounting medium) by setting the size parameters of the Analyze Particles tool from 30-infinity.

I did not count DCX+ cells in all images collected; instead sampling from a subset of images per bird. I counted one image of each ROI in each division (i.e., 3 images per ROI per bird, 1 each per rostral, mid, and caudal), similar to Guigueno et al. (2016). Generally, the image I counted cells from was the middle section (i.e., third) of the 5 sections imaged per division; in instances where the third section was damaged or torn, I counted cells from one of the adjacent sections (i.e., second or fourth). Overall, I counted cells in 539 images (n=403 excluding data from juveniles) using the Cell Counting plug-in, exhaustively counting all DCX+ cells in the image for which a distinct nucleus was visible. I performed separate counts of the three distinct morphologies of DCX+ cells described by Hall et al. (2014a) and Taufique et al. (2018): multipolar, fusiform, and hippocampal spherical (hereafter referred to as "round"). Figure 6 shows a representative example of the three morphologies of DCX+ cells I counted in this study. Different studies report varying DCX+ cell morphologies and categories; while many studies only count multipolar and fusiform cells (i.e., omitting round cells as a separate category; e.g., Balthazart et al., 2008), some studies focusing on DCX-ir specifically within the hippocampus tend to also count (or at minimum, acknowledge the presence of) round cells (e.g., Hall et al., 2014a; Lynch, 2018; Taufique et al., 2018), and indeed some have suggested it is the predominant cell type in this region (Hall et al., 2014a; Lynch, 2018).

2.2.11 STATISTICAL ANALYSES

All analyses were conducted using jamovi (version 2.2.5), an open-access statistical programming software integrated with R. All data were analyzed with linear models using the GAMLj (General Analyses for Linear Models in jamovi; version 2.6.5) module, using different models based on the type of data being analyzed. I screened data for normality and homogeneity of variances prior to analysis and transformed data where necessary (described below). Significance was set at $\alpha = .05$. Unless otherwise indicated, all post-hoc tests of main effects consisted of multiple pairwise comparisons; all p-values

derived from multiple pairwise comparisons were adjusted using the Bonferroni correction (denoted as p_B). I probed interaction effects using simple effects tests. Effect sizes are included for all models where such effect sizes were available. I did not exclude outliers from any analyses. Data were either visualized in RStudio (version 2022.02.3, running R, version 4.2.0) or directly in jamovi. Unless otherwise indicated, all data are presented as mean ± bootstrapped 95% confidence interval (CI).

2.3 RESULTS

2.3.1 GONADS

Gonad data from males and females were analyzed separately. I combined gonad data from photosensitive A (December) and photosensitive B (January) birds into one photosensitive group; as I did not expect reproductive condition to differ between birds captured one month apart in winter (under short day lengths; see Figure 2). In males, I first compared raw testis volume to the 20 mm^3 threshold for photostimulation described by Phillmore et al. (2006); six of the eight males in the photostimulated group did not meet this threshold (i.e., had testes < 20 mm^3), as shown in Figure 7A.

I tested for seasonal differences in testis volume using a general linear model; testis volume (natural-log transformed) was the dependent variable and photoperiodic condition was the fixed factor. There was a main effect of condition on testis volume ($F_{2,14}$=8.07, p=.005, ω^2=.454). Post-hoc tests showed testis volume in photostimulated males was significantly larger than photosensitive males (t_{14}=3.949, p_B=.004), but was not different from photorefractory birds (t_{14}=1.917, p_B=.228), as shown in Figure 7B.

Due to the ordinal nature of the scale used to assess ovary development in female black-capped chickadees (Table 3), I analyzed female gonad data using a non-parametric Kruskal-Wallis analysis of variance (as in Martin et al., 2020). Ovary stage was the dependent variable and photoperiodic condition was the grouping variable. While there was a main effect of photoperiodic condition on ovary stage (χ^2_2=7.67, p=.022), probing the effect further using Dwass-Steel-Critchlow-Fligner pairwise comparisons did not yield any significant differences (all ps > .05). Across all groups, only one female (captured in the photostimulated group) had stimulated ovaries (defined here as having an ovary score ≥ 3); all other females had ovary scores of 1 or 2, as shown in Figure 7C.

2.3.2 HEMATOCRIT

Hematocrit data were analyzed using a linear mixed model as there were multiple data points (i.e., microcapillary tubes) per bird. Prior to analysis, I excluded data from tubes containing small amounts of blood (total tube contents ≤ 5 on microhematocrit reader). Hematocrit fraction was the dependent variable and fixed factors were

photoperiodic condition, sex, and time of collection (i.e., field blood collected within 3 minutes of capture versus trunk blood collected immediately prior to perfusion). Bird ID was included as a random factor. There were no main effects of condition ($F_{3,19.1}$=.20, p=.895), sex ($F_{1,18.6}$=.01, p=.921), or time of collection ($F_{1,65.8}$=2.634, p=.109) and there were no significant interactions (Figure 8).

2.3.3 BRAIN WEIGHTS

I analyzed brain weight data with a linear mixed model, as there were multiple brain weight measures per bird. Brain weight was the dependent variable and the fixed factors were processing stage (post-perfusion/post-fix/post-cryoprotection), photoperiodic condition, and sex. Bird ID was included as a random factor. There was no main effect of photoperiodic condition ($F_{3,26}$=.806, p=.502) on brain weight, but there was a significant main effect of processing stage ($F_{2,52}$=521.398, p<.001) and sex ($F_{1,26}$=9.81, p=.004), as well as a condition × processing stage interaction ($F_{6,52}$=4.399, p=.001). Post-hoc tests showed post-fix brains were heaviest, post-perfusion brains were intermediate weight and post-cryoprotect weight were lightest; with each significantly differing from one another (all p_Bs<.001). Brains collected from male birds were also significantly heavier than brains collected from female birds (t_{26}=3.13, p_B=.004). Post-hoc analysis of the condition × processing stage interaction revealed that while cryoprotected brains were significantly lighter than post-perfusion brains in the photostimulated (t_{52}=8.7859, p_B<.001), photorefractory (t_{52}=5.6273, p_B<.001), and photosensitive B groups (t_{52}=3.75, p_B=.029), there was no significant difference in weight between post-perfusion and post-cryoprotected brains in the photostimulated A group (t_{52}=2.43, p_B=1), as shown in Figure 9.

2.3.4 BRAIN MORPHOMETRY: VOLUME

Volume data were analyzed using general linear models. I examined telencephalon volume and hippocampus volume separately. In each model, volume (either of telencephalon or hippocampus) was the dependent variable and fixed factors were photoperiodic condition and sex. I controlled for individual variation in telencephalon volume by including tarsus length and wing length as covariates. I

controlled for individual variation in hippocampal size by using telencephalon volume (minus hippocampal volume) as a covariate. No covariate interactions are included in any models. Anatomical and neurobiological variables were natural-log-transformed prior to analyses.

2.3.4.1 TELENCEPHALON VOLUME

There was a main effect of photoperiodic condition on telencephalon volume ($F_{3,26}$=3.93, p=.019, ω^2=.197); there was no main effect of sex ($F_{1,26}$=1.43, p=.243, ω^2=.010) nor a condition × sex interaction ($F_{3,26}$=1.39, p=.138, ω^2=.026). Post-hoc pairwise analyses of the main effect of condition revealed birds in the photosensitive B group had a smaller telencephalon than birds in the photosensitive A group (t_{26}=3.37, p_B=.014), as shown in Figure 10. No other pairwise comparisons were significant.

2.3.4.2 HIPPOCAMPAL VOLUME

There was no main effect of photoperiodic condition ($F_{3,27}$=.443, p=.724, ω^2=0), nor sex ($F_{1,27}$=1.901, p=.179, ω^2=.023) on hippocampal volume, as shown in Figure 11A. There was also no condition × sex interaction ($F_{3,27}$=1.159, p=.344, ω^2=.012). I also examined hippocampal volume as a proportion of telencephalon volume; and found no main effect of condition ($F_{3,28}$=.914, p=.447, ω^2=0), as shown in Figure 11B. There was also no main effect of sex ($F_{1,28}$=.380, p=.543, ω^2=0) nor an interaction ($F_{3,28}$=.226, p=.877, ω^2=0).

2.3.5 NEUROGENESIS: DCX+ CELL COUNTS

2.3.5.1 OVERALL REGIONAL DIFFERENCES IN DCX+ CELL TYPES

I first used a generalized linear model to examine whether the proportion of each DCX+ cell type (multipolar/fusiform/round) differed among ROIs. I calculated proportion of each cell type by dividing the number of cells of one cell type by the total number of cells counted in each FOV. Proportion data were fit to a binomial distribution and were analyzed using a generalized linear model (logit link function). Proportion was the dependent variable; region and cell type were fixed factors. Proportion data was significantly predicted by cell type (χ^2_2=819.24, p<.001), but not region (χ^2_3=5.46,

p=.141); there was also a significant region × cell type interaction (χ^2_6=79.33, p<.001). This indicated that while the overall proportion of each cell type does not vary across the four brain regions, there are significant differences in the relative proportions of each cell type between regions.

Further probing of this interaction with simple effects revealed the respective proportions of multipolar, fusiform, and round cell types did not significantly differ between hippocampal subregions HP1 and HP2 (multipolar: z=.729, p=.466; fusiform: z=.287,p=.774; round: z=.666, p=.505), but did significantly differ between the hippocampus and HA (multipolar: z=2.314, p=.021; fusiform: z=2.692,p=.007; round: z=3.795, p<.001), and between the hippocampus and LNP (multipolar: z=2.476, p=.013; fusiform: z=2.670, p=.008; round: z=3.957, p<.001). In other words, while round cells were the most abundant cell type across all four regions, they made up a significantly greater proportion of total cells in both hippocampal subregions (HP1/HP2) compared to control regions (HA, LNP); control regions were consequently comprised of a greater relative proportion of multipolar and fusiform cells (see Figure 12).

I subsequently analyzed raw count data for each cell type in each ROI separately. Originally, I fit raw count data to a Poisson distribution, however in all cases, count data were extremely overdispersed (dispersion parameter $\varphi \gg 3$), indicating the observed variance was greater than the assumed variance included in the model. Ignoring overdispersion increases type I error rate and may lead to inaccurate statistical inferences and conclusions (e.g., Cox, 1983; Ryan et al., 2021); I therefore opted to fit count data to a negative binomial distribution instead. This resolved the issue of overdispersion in all models ($\varphi \approx 1$). For each model, cell count was the dependent variable and fixed factors were photoperiodic condition and sex. I did not include axis as a factor in these models as I only counted cells in one image (of the five I captured) per rostral, mid, and caudal section.

2.3.5.2 REGIONAL DIFFERENCES IN DCX+ MULTIPOLAR CELLS

2.3.5.2.1 DCX+ MULTIPOLAR CELLS – HP1

There was no effect of photoperiodic condition (χ^2_3=2.45, *p*=.484; Figure 13, top left) nor sex (χ^2_1=1.95, *p*=.162) on multipolar cell counts in HP1. There was no interaction effect (χ^2_3=6.40, *p*=.094).

2.3.5.2.2 DCX+ MULTIPOLAR CELLS – HP2

There was no effect of photoperiodic condition (χ^2_3=4.682, *p*=.197; Figure 13, top right) nor sex (χ^2_1=.335, *p*=.562) on multipolar cell counts in HP2. There was a significant condition × sex interaction (χ^2_3=8.833, *p*=.032), however when the interaction effect was probed using simple effects (i.e., examining seasonal differences in each sex separately), no significant differences were observed (females: χ^2_3=4.51, *p*=.211; males: χ^2_3=7.13, *p*=.068).

2.3.5.2.3 DCX+ MULTIPOLAR CELLS – HA

There was no effect of photoperiodic condition (χ^2_3=4.580, p=.205; Figure 13, bottom left) nor sex (χ^2_1=.259, *p*=.611) on multipolar cell counts in HA. There was no interaction effect (χ^2_3=.595, *p*=.898).

2.3.5.2.4 DCX+ MULTIPOLAR CELLS – LNP

There was no effect of photoperiodic condition (χ^2_3=3.007, *p*=.391; Figure 13, bottom right) nor sex (χ^2_1=3.175, *p*=.877) on multipolar cell counts in LNP. There was no interaction effect (χ^2_3=.643, *p*=.887).

2.3.5.3 REGIONAL DIFFERENCES IN DCX+ FUSIFORM CELLS

2.3.5.3.1 DCX+ FUSIFORM CELLS – HP1

There was no effect of photoperiodic condition (χ^2_3=.868, *p*=.833; Figure 14, top left) nor sex (χ^2_1=.328, *p*=.567) on fusiform cell counts in HP1. There was no interaction effect (χ^2_3=2.017, *p*=.569).

2.3.5.3.2 DCX+ FUSIFORM CELLS – HP2

There was no effect of photoperiodic condition (χ^2_3=1.254, p=.740; Figure 14, top right) nor sex (χ^2_1=.354, p=.552) on fusiform cell counts in HP2. There was no interaction effect (χ^2_3=.151, p=.985).

2.3.5.3.3 DCX+ FUSIFORM CELLS – HA

There was no effect of photoperiodic condition (χ^2_3=3.67, p=.299; Figure 14, bottom left) nor sex (χ^2_1=1.19, p=.276) on fusiform cell counts in HA. There was no interaction effect (χ^2_3=1.10, p=.776).

2.3.5.3.4 DCX+ FUSIFORM CELLS – LNP

There was a main effect of photoperiodic condition on fusiform cell counts in LNP (χ^2_3=9.94, p=.019). There was no main effect of sex (χ^2_1=3.49, p=.062), nor an interaction effect (χ^2_3=5.59, p=.133). Post-hoc pairwise analysis of the main effect of condition showed birds in the photosensitive A group had significantly fewer fusiform cells in LNP compared to birds in the photostimulated group (z=2.972, p_B=.018) and the photorefractory group (z=2.823, p_B=.029), as shown in Figure 14 (bottom right).

2.3.5.4 REGIONAL DIFFERENCES IN DCX+ ROUND CELLS

2.3.5.4.1 DCX+ ROUND CELLS – HP1

There was no effect of photoperiodic condition (χ^2_3=3.26, p=.353) nor sex (χ^2_1=1.54, p=.215) on round cell counts in HP1. There was however a significant condition × sex interaction (χ^2_3=18.01, p<.001). When probing the interaction using simple effects (i.e., examining seasonal differences in each sex separately), analyses revealed a significant difference in HP1 round cells across photoperiodic condition in females (χ^2_3=15.50, p=.001), but not males (χ^2_3=5.44, p=.143), as shown in Figure 15 (HP1 panel; top). Photosensitive females had more round cells than photostimulated females (z=2.245, p=.025) and photorefractory females (z=2.408, p=.016); the two photosensitive groups did not differ between each other (z=.203, p=.839).

2.3.5.4.2 DCX+ ROUND CELLS – HP2

There was no effect of photoperiodic condition (χ^2_3=2.80, p=.424) nor sex (χ^2_1=1.79, p=.181) on round cell counts in HP2. There was however a significant condition × sex interaction (χ^2_3=16.48, p<.001). As above, I probed the interaction using simple effects, examining seasonal differences in each sex separately. Analyses revealed significant differences in HP2 round cells across photoperiodic condition in both females (χ^2_3=8.94, p=.03) and males (χ^2_3=10.30, p=.016), as shown in Figure 15 (HP2 panel; second from top). In females, photosensitive females had more round cells than photorefractory females (z=2.69, p=.007), but not photostimulated females (z=1.17, p=.242); the two groups of photosensitive females did not differ from each other (z=.054, p=.957). In males, photosensitive A males had more round cells than photosensitive B males (z=2.421, p=.015), but did not differ from photostimulated (z=.0642, p=.949) or photorefractory males (z=1.1303, p=.258).

2.3.5.4.3 DCX+ ROUND CELLS – HA

There was no effect of photoperiodic condition (χ^2_3=5.242, p=.155) nor sex (χ^2_1=.735, p=.391) on round cell counts in HA. There was a significant condition × sex interaction (χ^2_3=13.16, p=.004). Probing the interaction using simple effects revealed a significant difference in HA round cell counts across photoperiodic condition in females (χ^2_3=17.39, p<.001), but not males (χ^2_3=3.28, p=0.351), as shown in Figure 15 (HA panel; second from bottom). Photosensitive females had more round cells than photorefractory females (z=2.824, p=.005); but not photostimulated females (z=1.937, p=.053). The two photosensitive groups did not differ between each other (z=.457, p=.648).

2.3.5.4.4 DCX+ ROUND CELLS – LNP

While there was no effect of photoperiodic condition (χ^2_3=2.91, p=.406) on round cell counts in LNP, there was a main effect of sex (χ^2_1=6.41, p=.011) and a significant condition × sex interaction (χ^2_3=24.38, p<.001). I did not directly examine the main effect of sex; opting instead to probe the condition × sex interaction. Simple effects analyses revealed significant differences in LNP round cells across photoperiodic condition in both females (χ^2_3=18.68, p<.001) and males (χ^2_3=8.41, p=.038), as illustrated in Figure 15

(LNP panel; bottom). In females, photosensitive birds had more round cells than photostimulated (z=2.1076, p=.035) and photorefractory birds (z=3.0367, p=.002); the two photosensitive groups did not differ from each other (z=.0519, p=.959). In males, photosensitive males had significantly fewer round cells in LNP compared to photorefractory males (z=2.1911, p=.028), but not photostimulated males (z=.1519, p=.879); the two photosensitive groups did not differ between each other (z=1.0596, p=.289).

2.3.6 NEUROGENESIS: %DCX+ COVERAGE

I analyzed %DCX+ coverage data using linear mixed models, as there were multiple coverage measurements per ROI per bird. Data were arcsine-square root-transformed prior to analyses.

2.3.6.1 OVERALL REGIONAL DIFFERENCES IN %DCX+ COVERAGE

I first examined overall differences in %DCX+ coverage between ROIs. In this model, transformed %DCX+ coverage was the dependent variable, and ROI (HA/HP1/HP2/LNP) was the fixed factor. Bird ID was included as the random factor. There was a main effect of region on %DCX+ coverage ($F_{3,1830}$=270, p<.001), as shown in Figure 12. Post-hoc pairwise comparisons revealed significant differences in %DCX+ coverage among all ROIs (p_{BS}<.001) except for between the two hippocampus ROIs (HP1-HP2: t_{1829}=1.57, p=.699). Coverage was highest in the two hippocampal ROIs; coverage in HA was also higher than coverage in LNP (Figure 16). I therefore completed all subsequent coverage analyses for each region using separate linear mixed models, where %DCX+ coverage was the dependent variable, and photoperiodic condition, sex, and axis (rostral/mid/caudal) were the fixed factors. Bird ID was included as the random factor.

2.3.6.2 %DCX+ COVERAGE IN HP1

There was no effect of photoperiodic condition ($F_{3,28.3}$=.684, p=.569) on %DCX+ coverage in HP1. There was however a main effect of sex ($F_{1,28.2}$=4.431, p=.044) and axis ($F_{2,430.5}$=12.576, p<.001), and a significant condition × axis interaction effect ($F_{6,430.4}$=4.341, p<.001). No other interactions were significant. Post-hoc pairwise

analysis of the main effect of sex showed male birds had a higher %DCX+ coverage in HP1 overall compared to females ($t_{27.7}$=2.1, p_B=.045). I did not examine the main effect of axis; opting instead to probe the condition × axis interaction effect using simple effects, testing only for differences between rostral and caudal sections. %DCX+ coverage in HP1 was consistently higher in the rostral than caudal division across all photoperiodic conditions (photosensitive A: t_{428}=3.342, p<.001; photosensitive B: t_{439}=2.509, p=.012; photorefractory: t_{430}=2.736, p=.006) except for in the photostimulated group (t_{430}=.923, p=.357), as shown in Figure 17 (HP1 panel; top).

2.3.6.3 %DCX+ COVERAGE IN HP2

There was no effect of photoperiodic condition ($F_{3,28.2}$=.356, p=.785) nor sex ($F_{1,28.2}$=.838, p=.368) on DCX+ coverage in HP2. There was however a main effect of axis ($F_{2,416.3}$=4.274, p=.015) and a significant condition × axis interaction ($F_{6,416.2}$=2.211, p=.041). No other interactions were significant. As above, I did not examine the main effect of axis; opting instead to probe the condition × axis interaction effect using simple effects, testing only for differences between rostral and caudal sections. In contrast to HP1, analyses showed no rostral-caudal differences in %DCX+ coverage in HP2 across all photoperiodic conditions except for in the photostimulated group (Figure 17; HP2 panel; second from top), where there was significantly higher %DCX+ coverage in the caudal section of HP2 compared to the rostral section (t_{416}=2.337, p=.02).

2.3.6.4 %DCX+ COVERAGE IN HA

There was no effect of photoperiodic condition ($F_{3,31.2}$=1.478, p=.24) nor sex ($F_{1,32.9}$=2.132, p=.154) on DCX+ coverage in HA. There was however a main effect of axis ($F_{2,416.3}$=4.274, p=.015) and a significant condition × axis interaction effect ($F_{6,366.1}$=.653, p=.007). No other interactions were significant. As in the hippocampus ROIs, I did not examine the main effect of axis; opting instead to probe the condition × axis interaction. Simple effects tests revealed no rostral-caudal differences in %DCX+ coverage in HA across any photoperiodic condition except for the photosensitive A group (Figure 17; HA panel; second from bottom), where there was significantly higher

%DCX+ coverage in the caudal section of HA compared to the rostral section (t_{362}=4.27, p<.001).

2.3.6.5 %DCX+ COVERAGE IN LNP

There was no effect of photoperiodic condition ($F_{3,27.9}$=.725, p=.546) nor sex ($F_{1,27.9}$=.157, p=.695) on DCX+ coverage in HA. There was a main effect of axis ($F_{2,468.5}$=12.179, p=.015). Unlike in other ROIs, there was no significant condition × axis interaction ($F_{6,468.4}$=.344, p=.913). I therefore examined the main effect of axis using multiple pairwise comparisons; analyses showed that across photoperiodic conditions, there was significantly less %DCX+ coverage in the caudal portion of LNP relative to the mid LNP (t_{470}=4.27, p_B<.001) and rostral LNP (t_{470}=4.36, p_B<.001), as shown in Figure 17 (LNP panel; bottom).

2.4 DISCUSSION

The aim of this study was to further characterize seasonal changes in hippocampal neural plasticity in black-capped chickadees, based on their physiological photoperiodic condition, while also minimizing the potentially confounding effects of captivity. Although hippocampal volume did not vary seasonally, there was some evidence of seasonal hippocampal plasticity in hippocampal neurogenesis. However, patterns of the seasonal variation in hippocampal neurogenesis appears to vary between the sexes: in females hippocampal neurogenesis peaks in January, while males generally show no change over the year. There was also some sex-specific seasonal variation in neurogenesis in the two non-hippocampal control regions sampled (hyperpallium apicale, HA; lateral nidopallium, LNP).

2.4.1 PHYSIOLOGICAL MEASURES

2.4.1.1 GONADS

Instead of defining seasonality in terms of month captured (e.g., Barnea and Nottebohm, 1994; Hoshooley et al., 2007; Lange et al., 2021) or season captured (e.g., Hoshooley and Sherry, 2007), the seasonal groups in this study were based on predicted photoperiodic condition (breeding state). The photostimulated group was captured in March-April, the photorefractory group was captured in August-September, and photosensitive birds were captured in December-January. I predicted that birds in each group would display the gonadal characteristics associated with that photoperiodic condition: developed or recrudesced gonads in photostimulated birds (indicative of breeding condition) and regressed gonads (indicative of a non-breeding state) in photorefractory and photosensitive birds.

Gonad data revealed males and females in the photorefractory and photosensitive groups had regressed gonads (i.e., testis volume $\ll 20$ mm^3; ovary stage = 1 or 2), indicating a non-breeding state, as expected. However, gonad data from both males and females in the predicted photostimulated group (captured in March-April) indicated that very few subjects showed the typical gonadal indicators of photostimulation. Only 2 of the 8 males in the photostimulated group met the 20 mm^3 standard for photostimulation

described by Phillmore et al. (2006; Figure 7A), suggesting most males captured were not in peak breeding condition. There was some evidence that photostimulated birds, particularly males, were at least partially photostimulated, as photostimulated males had significantly larger testes compared to photosensitive males (captured in December-January), however, testis volume did not vary significantly between photostimulated and photorefractory males (captured in August-September; Figure 7B). Only 1 of the 8 females in the photostimulated group had stimulated ovaries (i.e., ovary stage ≥ 3); stage of ovary development did not significantly differ across groups (Figure 7C), indicating no differences in breeding condition across groups. Taken together, these data suggest that the timeframe used to capture photostimulated birds (25 March-10 April) was generally insufficient to capture mostly photostimulated birds, at least if only using gonad condition (i.e., testis volume in males/ovary stage in females) to define breeding condition.

Photostimulation is not a binary process; activation of the HPG axis only occurs when day length becomes sufficiently long in spring, and even after the photoperiodic threshold ("critical day length") for initial photostimulation is met, the cascading endocrine, physiological, and behavioural effects that occur in response to photostimulation are not immediate (Nicholls et al., 1988). Given that some birds (a small group of males) in the spring group showed evidence of photostimulation, it is unlikely that the capture window entirely preceded the critical day length threshold for initial photostimulation. It is much more likely that birds were captured too early in the season – that is, not enough time had elapsed between the threshold for initial photostimulation and the time in which most chickadees begin to display the complete physical characteristics of breeding.

Nicholls et al. (1988) estimate the time between the critical day length threshold for photostimulation and peak reproductive ability to be ca. 3 to 4 weeks, and while this can vary greatly between species and geographic regions (reviewed in Hahn and MacDougall-Shackleton, 2008), when comparing the capture dates in this study to previous seasonal studies of black-capped chickadees carried out in the same region as this study, we see that even a 2 week delay between capture dates can yield markedly different findings. For example, Phillmore et al. (2011) and Phillmore et al. (2015)

captured adult black-capped chickadees to investigate seasonal changes in the expressions of ZENK and FOXP2 (respectively); breeding birds collected for both studies were captured between April 18-May 15 (i.e., capturing began ca. 2-3 weeks later than this study), and of the 16 males captured for these studies, 15 were photostimulated (i.e., testis volume > 20 mm^3).

Given that that photostimulated birds were not a truly representative sample of birds in full breeding condition, I describe all subsequent findings in the context of the months in which birds were captured, for ease of discussion purposes.

2.4.1.2 HEMATOCRIT

I found no evidence of seasonal or sex differences in hematocrit in black-capped chickadees captured in this study (Figure 8). While these data suggest hematocrit does not vary with season or sex in this species, given that so few of the birds in the photostimulated group did not meet the established gonadal standards for breeding condition, I cannot reasonably conclude whether hematocrit levels vary (or do not vary) with reproductive state. The hematocrit values observed across the year in this sample of black-capped chickadees (ca. 45-50%; Figure 8) are generally consistent with values observed in prior studies of black-capped chickadees (e.g., van Oort et al., 2007; Proppe et al., 2013; Petit et al., 2017); to my knowledge, no study has found variation in hematocrit with season or sex in this species. However, van Oort et al. (2007) did find variation in hematocrit with dominance hierarchy status in breeding male black-capped chickadees, showing dominant males during the breeding season had higher hematocrit than subordinate males. Dominance hierarchies may potentially be a driver of the trends in hippocampal neurogenesis observed in winter, particularly in females (described below, section 2.4.2.2); therefore further investigation on this topic may be warranted. However, there is significant debate in the literature as to whether hematocrit is indeed a true and reliable indicator of physiological condition in wild birds (reviewed in Fair et al., 2007).

2.4.1.3 BRAIN WEIGHT

Upon extraction from the skull, brain tissue is placed first in a fixative (e.g., paraformaldehyde) for a specified period (usually on the order of days to weeks); fixatives reduce potential background "noise" in brain tissue from non-neural artifacts (e.g., blood vessels) which may interfere with histological or immunohistochemical staining (Roth et al., 2010). Samples are then transferred from fixative to a cryoprotective solution (usually high concentrations of sucrose or ethylene glycol in buffer); cryoprotection prior to long-term storage at low temperatures (e.g., -80ºC; as in this study) prevents the accumulation of ice crystals which can damage tissue and produce holes (known as "Swiss cheese artifacts"; Scouten, 2010) in the samples. However, treating tissue specimens with high concentrations of sucrose for cryoprotective purposes also alters the tissue weight via its effects on tissue water content, usually by making specimens lighter than they were post-extraction (i.e., shrinking the samples; Smulders, 2002).

Further, both Smulders (2002) and Phillmore et al. (2006) have shown brain shrinkage as a result of cryoprotection can also vary seasonally in songbirds. Using data collected from Smulders et al. (1995; 2000), Smulders (2002) showed that in black-capped chickadees, brain weights post-perfusion and brain weights post-cryoprotection differed significantly in birds captured in April and June, but not in August, October or December. Likewise, Phillmore et al. (2006), using brain weight data from the same birds used to study seasonal hippocampal neurogenesis in Hoshooley et al. (2007), found evidence of significant brain shrinkage in birds captured in July, but not in April, January, and October.

Here, I confirm both the findings of Smulders (2002) and Phillmore et al. (2006) in this sample (Figure 9): there was significant shrinkage between perfusion and cryoprotection in brains collected in March-April (as in Smulders, 2002); as well as in brains collected in August-September (similar to Phillmore et al., 2006). One of the findings from this study do however differ from both Smulders (2002) and Phillmore et al. (2006): in addition to finding shrinkage in the spring and late summer, I also report evidence of shrinkage in the brains of birds captured in January. Further, I did not find evidence of shrinkage in brains captured in December, similar to both prior studies.

The overall mechanisms associated with seasonal differences in brain water content (and by extension, tissue shrinkage during cryoprotection) remain purely speculative at this point (reviewed in Smulders, 2002), but may be a result of seasonal differences in glucocorticoid-mineralocorticoid interactions in the brain. This may warrant further sturdy, particularly given the atypical winter peak in glucocorticoids observed in some food-storing species (e.g., Silverin, 1997; Silverin, 1998; Pravosudov, 2007) and the interaction between seasonality, stress, and hippocampus-dependent spatial memory (reviewed in LaDage, 2015).

2.4.2 VOLUMETRIC DIFFERENCES

There was some evidence of seasonal variation in telencephalon size in black-capped chickadees: birds captured in January had a significantly smaller telencephalon than birds captured in December; no other differences between groups were detected (Figure 10). However, in contrast to the patterns observed in the neurogenesis data, no evidence of seasonal variation in actual or relative hippocampal volume was found in this sample of black-capped chickadees (Figure 11). These results differ from Smulders et al. (1995), who found a peak in hippocampal volume in October, as well as to Hoshooley and Sherry (2007), who found a peak in hippocampal volume in spring.

This is perhaps not surprising: prior studies have found differences in hippocampal neurogenesis across seasons without detecting a difference in overall (or relative) hippocampal volume. Indeed no seasonal study of black-capped chickadees to my knowledge has found concurrent peaks in hippocampal volume and neurogenesis. Those studies which examine both measures of plasticity typically find an effect of season in one measure, but not the other. For example, Hoshooley et al. (2007), who found a January peak in neurogenesis in the chickadee hippocampus, did not detect any significant differences in hippocampal volume across seasons. Further, Guigueno et al. (2016) found seasonal and sex differences in neurogenesis in brown-headed cowbirds but did not detect any seasonal or sex differences in hippocampal volume.

The validity of volume as a reliable measure of plasticity has been brought into question; some have suggested that differences between research groups in the histological techniques used to quantify telencephalon and hippocampal volume may be a

contributor to the variation in findings on seasonal volumetric change in chickadees (e.g., Smulders, 2002; Roth et al., 2010; Pravosudov et al., 2015); this may also be a reason why differences in neurogenesis can be detected without necessarily detecting a volumetric difference. Perhaps it is volume alone that is the issue, and to therefore understand the mechanisms underlying seasonal volume change evident in some studies (e.g., Smulders et al., 1995; Hoshooley and Sherry, 2007; Lange et al., 2021), perhaps multiple measures of plasticity, including volume, neuron number, and neurogenesis, require concurrent examination. Other measures of plasticity, including apoptosis (e.g., Hoshooley and Sherry, 2004), soma size (e.g., Freas et al., 2013a, Freas et al., 2013b) and dendritic architecture (e.g., Roth et al., 2017) may also play a role in uncovering the mechanisms underlying neural plasticity which may contribute to volume change. Such an approach would therefore allow researchers to discern why volumetric change may occur in some circumstances, but not others.

2.4.3 NEUROGENESIS – DCX+ CELL COUNTS

I quantified neurogenesis by examining seasonal and sex differences in the number and proportion of immature neurons expressing DCX. I counted three previously identified DCX+ cell morphologies: multipolar, fusiform, and round (as in Hall et al., 2014a; Taufique et al., 2018; Lynch, 2018). There was no evidence of seasonal variation or sex differences in the number of multipolar or fusiform DCX+ cells in the hippocampus of black-capped chickadees (see Figures 13-14). However, there was seasonal variation in the number of DCX+ round cells, a morphology of immature neuron that appears to be principal DCX+ cell type in the hippocampus (Hall et al., 2014a; Lynch, 2018), but less-well described and understood in the literature.

2.4.3.1 THE ROLE OF DCX+ ROUND CELLS IN THE AVIAN HIPPOCAMPUS

In previous studies, DCX+ round cells have been described as the predominant cell type in the hippocampus of several species, including European starlings (Hall et al., 2014a), Indian house crows (*Corvus splendens*; Taufique et al., 2018) and brown-headed cowbirds (Lynch, 2018). However, it is not clear whether this specific DCX+ cell type was observed in other regions in smaller numbers. Here, I confirm that round cells make

up the vast majority (≥ 95%) of DCX+ cells in the hippocampus of black-capped chickadees, in significantly greater proportions than control regions, including the nearby structure HA and the distant structure LNP (Figure 12). While round cells were found to be the predominant cell type across all ROIs (hippocampus, HA, LNP), HA and LNP contain round cells in significantly smaller proportions than the hippocampus.

Similar to Taufique et al. (2018) and Lynch (2018), but unlike Hall et al. (2014a), a small but consistent proportion of multipolar and fusiform cells were also observed in the chickadee hippocampus (Figure 12). The differences between multipolar and fusiform DCX+ cells are purportedly driven by development: fusiform cells are immature neurons actively migrating towards their site of recruitment, whereas multipolar cells have already arrived at their site of recruitment and are in the process of being functionally incorporated into circuits within that region (Balthazart et al., 2008). However, given the lack of study around DCX+ round cells, the potential functional roles and/or developmental timelines associated with this particular cell type, especially relative to multipolar and fusiform cells, are not well-understood.

So, what role might these DCX+ round cells play in the songbird brain? Given the high proportion of round cells in the hippocampus observed here in black-capped chickadees as well as in other species, it is possible that round cells specifically support spatial behaviour. However, the presence of round cells in the hippocampus of European starlings (generally considered to be a non-specialist species) suggest that these round cells are likely not specific to any particular spatial behaviour (e.g., food-storing), but rather support more broad spatial behaviours exhibited across many avian taxa (e.g., navigation during flight). Round cells may also simply be the hippocampus-equivalent of multipolar cells (as speculated by Hall et al., 2014a): that is, some factor(s) specifically expressed by other cells in the hippocampus (e.g., differences in signalling cues or glial interactions; as in rodents, Ashton et al., 2012; Parkitny and Maletic-Savatic, 2021) lead to immature neurons not requiring the same morphological characteristics of multipolar cells in other brain regions (e.g., multiple long processes) for successful integration into pre-existing hippocampal circuits, hence these cells adapt a simple “round” morphology. However, if these were the case, it would still not explain why some proportion of

"classical" DCX+ multipolar cells (albeit a rather small one, ca. 5%; Figure 12) were found in the chickadee hippocampus.

Ultimately, much more work is needed to truly understand the functional roles of DCX+ immature neurons, including the lesser known (but abundant) round cell in the songbird brain; future studies should continue to examine how these DCX+ round cells contribute to other manifestations of hippocampal plasticity in other spatial specialists (e.g., brood parasites, long-distance migrants).

2.4.3.2 SEX AND SEASONAL VARIATION IN NEUROGENESIS IN BLACK-CAPPED CHICKADEES

While minimal variation in hippocampal neurogenesis was found across the year in male black-capped chickadees, there was evidence of seasonal variation in hippocampal neurogenesis in female black-capped chickadees: specifically, females captured in winter (December/January) had significantly more DCX+ round cells in the hippocampus compared to females captured in spring (March-April; subregion HP1 only) or late summer (August-September; both HP1 and HP2), as shown in Figure 15. The findings in females in this study are generally in line with those of Hoshooley et al. (2007), who also showed a peak in hippocampal neurogenesis (measured using BrdU) in chickadees captured in January; these findings differ from Barnea and Nottebohm (1994), who showed a peak in neurogenesis (measured using [^{3}H]-TdR) in chickadees captured in October. These findings also lend support to the notion that increased hippocampal neurogenesis in winter is a result of an increased demand on memory while birds are actively recalling the location of their food stores during retrieval (as suggested by Hoshooley et al., 2004; Sherry and Hoshooley, 2010), as opposed to while storing in fall (as suggested by Barnea and Nottebohm, 1994; Smulders et al., 1995; 2000; Lange et al., 2021).

Female-biased sex differences in hippocampal neural plasticity (and neurogenesis) have been observed in other avian and non-avian species (reviewed in Lee et al., 1998; Sherry and Guigueno, 2019), however these sex differences in the brain (and how they may vary seasonally) are generally believed (or at minimum, speculated) to support and correspond to well-documented sex and seasonal differences in ecologically-

relevant spatial behaviour and/or memory by a particular species (e.g., Sherry et al., 1992; Lee et al., 1998; Jones et al., 2003). For example, in obligate brood parasites (e.g., brown-headed cowbirds), females, not males, are responsible for searching for and returning to "host" nests in which to lay their eggs (reviewed in Sherry and Guigueno, 2019); it is believed that a larger hippocampus and increased hippocampal neurogenesis observed in female cowbirds (e.g., Sherry et al., 1993; Guigueno et al., 2016) supports this female-biased difference in spatial behaviour.

The hippocampus-dependent behaviour I examined in this study (food storing and retrieval), while known to potentially vary subtly between male and female chickadees, is not sex-specific (e.g., Petersen and Sherry, 1996), thus I had no expectation there would be differences in hippocampal neurogenesis between males and females. Indeed, there is a considerable body of literature suggesting no sex differences in the chickadee hippocampus (e.g., Petersen and Sherry, 1996; Pravosudov and Clayton, 2002; Roth et al., 2011). For example, Petersen and Sherry (1996) showed no sex differences in telencephalon volume, hippocampal volume, food-storing behaviour, and spatial memory in male and female black capped chickadees. While Petersen and Sherry (1996) captured birds over several months (from October-March), they did not consider season (or month of capture) as a factor in their analyses, likely because birds were captured in 1992; pre-dating the discoveries of seasonal hippocampal plasticity by Barnea and Nottebohm (1994) and Smulders et al. (1995). Further, Petersen and Sherry (1996) examined both the brain and behaviour of chickadees kept in captivity. Captivity can drastically affect hippocampal volume in chickadees (reviewed in Phillmore et al., 2022), which is why I minimized the time between capture and sacrifice in this study. Similarly, food-storing behaviour in captivity is not comparable to food-storing behaviour observed in the wild. It is therefore possible that using captive birds, and not considering season as a factor, may have suppressed any potential sex differences.

Despite not finding any sex differences in their study, Petersen and Sherry (1996) still speculated that sex differences in food-storing behaviour and the hippocampus may still be present in wild black-capped chickadees, particularly in the fall and winter months, if one sex is more reliant on food stores as a result of either social (e.g., interactions within dominance hierarchies) or physiological (e.g., sex differences in body

weight or energy expenditure) pressures. Females do in fact occupy lower ranks than males in dominance hierarchies in winter flocks (e.g., Smith, 1991, Ratcliffe et al., 2007, Foote et al., 2020). Further, foraging and energy expenditure is known to vary with hierarchy rank in chickadees (e.g., Lewden et al., 2012), and known to vary seasonally in chickadees (e.g., Karasov et al., 1992; Cooper and Swanson, 1994; Cooper, 2000), but whether there are sex differences in energy expenditure in this species remains untested, a result of the difficulty associated with reliably confirming sex in the field observations (Cooper and Swanson, 1994). However, there is evidence to suggest that within winter dominance hierarchies females tend to be leaner than males (Ratcliffe et al., 2007), and it is generally accepted that male chickadees are larger overall than females (Foote et al., 2010 and citations; but see Robertson et al., 2020). Therefore, as Petersen and Sherry (1996) suggest, it is possible that the increased hippocampal neurogenesis observed in females, but not males, may be a result of the above mentioned social (i.e., dominance hierarchies) and physiological (i.e., body condition and weight) pressures forcing female black-capped chickadees to rely more heavily on food stores (and by extension, hippocampal-dependent spatial memory) than males; and this may be supported by an increase in hippocampal neurogenesis only in winter. Conversely, the reason that these same seasonal patterns were not observed in the male chickadees examined in this study may be that males do not experience these same social or physiological pressures (e.g., as a result of an increased rank in dominance hierarchies) that females do, and as a consequence, the demand on hippocampus-dependent spatial memory is relatively constant over the year.

However, studies that have directly compared male and female chickadee spatial behaviour generally do not show differences. Van Buskirk and Smith (1989) found no differences in several measures of foraging behaviour (including duration of foraging, flights per minute, locations of foraging sites) between male and female black-capped chickadees observed in the wild over the winter months (December-March). Further, Branch et al. (2020) found no overall differences in performance between male and female mountain chickadees on a spatial learning and memory task over a 4-year period. However, Branch et al. (2020) did detect a more subtle difference: males consistently exhibited a wider range of variation in task performance compared to females, and

females' performance on the spatial learning task correlated with their performance on a separate reversal spatial task; this effect was not observed in males. While performance on an artificial task indicated that the outward behaviours related to foraging do not differ, Branch et al. (2020) speculate that stronger natural selection for enhanced cognitive abilities in females of this species resulted in less variation in the task.

Enhanced cognitive abilities in female black-capped chickadees are also evident in studies of auditory perception; recent studies have shown female chickadees perform better and more accurately than males on acoustic discrimination tasks (e.g., Hoeschele et al., 2012; Roach et al., 2017; but see Charrier et al., 2005; Guillette et al., 2009). Further, in a parallel study to Roach et al. (2017), Roach et al. (2016) showed females exposed to the same acoustic stimuli presented in Roach et al. (2017) have increased activity (measured by the expression of the immediate early gene ZENK) in auditory perceptual regions caudomedial nidopallium (NCM) and caudomedial mesopallium (CMM) than males, suggesting these differences in performance on an auditory perceptual task may also be represented by differences in the brain.

2.4.3.3 CONTROL REGIONS

In addition to the hippocampus, seasonal variation in neurogenesis was also observed in the hyperpallium apicale (HA) and in the lateral nidopallium (LNP) of female chickadees (Figures 14-15). Both of these regions were sampled from as non-hippocampal control regions, and both were regions in which I did not anticipate finding any seasonal change.

2.4.3.3.1 HA

As in the hippocampus, female chickadees captured in winter (December/January) had significantly more DCX+ round cells in HA than females captured in fall (Figure 15; HA panel, second from bottom). This is in contrast to Hoshooley et al. (2005), who found more neurogenesis (measured by BrdU) in HA in photostimulated (i.e., spring birds) chickadees relative to photorefractory and photosensitive birds. However, Hoshooley et al. (2005) suggest that the spring peak in HA may not be a result of new neurons actually becoming integrated into this region but are rather in the process of migrating via HA to

nearby vocal region HVC (located directly ventral to HA) as a result of increased recruitment in spring to support an increase in vocal behaviour that occurs in spring.

Our findings show a peak in HA neurogenesis in females in winter, and while this is in contrast to Hoshooley et al. (2005), it is not entirely surprising, given that region HA has been recently implicated in spatial processing. While the presence of place cells in the avian brain has been hypothesized for many years (reviewed in Sherry et al., 2017), only recent advances in electrophysiological techniques have provided rigorous evidence for classical, mammalian-like place cells in the avian hippocampus (Payne et al., 2021; Agarwal et al., 2021), and, interestingly, also in HA (Agarwal et al., 2021). Given that Agarwal et al. (2021) has shown some degree of spatial coding occurring in HA (although this has not been extensively tested or examined in food-storing birds or songbirds, as their study species was the barn owl), while purely speculative, the same differences described above that may drive a winter peak in hippocampal neurogenesis in females may also contribute to the same seasonal patterns of neurogenesis observed in females (but not males) in the nearby region HA.

2.4.3.3.2 LNP

As shown in Figure 14 (bottom right panel), there was an overall seasonal difference in the number of DCX+ fusiform cells in the control region lateral nidopallium. Seasonal patterns of DCX+ round cells in LNP were also found to vary with sex (Figure 15; LNP panel, bottom right). I chose to sample from LNP because, as opposed to control region HA, it is anatomically distant from the hippocampus (Figure 5). To my knowledge, no study has previously examined patterns of neurogenesis in LNP in food-storing birds. There is some evidence from developmental studies of domesticated chickens (*Gallus gallus*) to suggest LNP may be involved in different types of social learning (e.g., imprinting; Salzen et al., 1975, Salzen et al., 1978). Some studies have found a role for the LNP in sexual imprinting in songbirds, primarily zebra finches (e.g., Bischof and Herrmann, 1986; Sadananda and Bischof, 2006), the overall role of this non-nuclear brain region in food-storing birds is largely unknown.

However, it is possible that the seasonal patterns of neurogenesis observed here can be attributed to seasonal variation in social interactions between male and female

black-capped chickadees. For example, the peak in fusiform cells observed across all birds in late winter, spring and summer (Figure 14) may be a result of pair-bonding (or preparation for pair bonding) and breeding; further, the winter peak in round cells observed in females, but not males (similar to that which was observed in the hippocampus and HA; Figure 15) may be a result of particular male-female interactions within dominance hierarchies. While these findings in LNP are unexpected and may have some implications in the context of social interactions (although this is purely speculative), further studies are necessary to properly examine patterns of neural plasticity in the lateral nidopallium of food-storing birds.

2.4.4 NEUROGENESIS – %DCX+ COVERAGE

I also quantified neurogenesis by examining the proportion of DCX-ir (%DCX+ coverage) per microscope field of view. %DCX+ coverage was significantly higher in the hippocampus relative to the two non-hippocampal control regions (Figure 16), but there were no main seasonal or sex differences in any of the four ROIs. However, across all ROIs, %DCX+ coverage varied along the rostral-caudal axis of the brain, and some of these rostral-caudal patterns of %DCX+ showed evidence of seasonal variation (Figure 17).

In the dorsolateral hippocampus (HP1) there was significantly greater %DCX+ coverage in the rostral portion of the brain compared to the caudal portion of the brain across all seasons, except for those birds captured in March-April (Figure 17; HP1 panel, top). The overall rostral-caudal difference is consistent with Barnea and Nottebohm (1994), who showed that rostral hippocampus of free-ranging black-capped chickadees contained significantly more new neurons (measured using [^{3}H]-TdR) than the caudal hippocampus. Further, Lynch (2018) found more DCX+ cells in the rostral hippocampus of brown-headed cowbirds compared to the caudal portion. This same pattern also appears to persist in the hippocampus of non-songbirds, with Mehlhorn et al. (2022) showing more DCX+ cells in the rostral portion of the pigeon hippocampus compared to the caudal section.

Interestingly, in HP1 it was found that males had more overall %DCX+ coverage in HP1 than females. While inconsistent with cell count data in either hippocampal

subregion, the lack of season × sex interaction suggests this sex difference may be simply attributable to males having an overall higher (but seasonally unchanging) rate of neurogenesis in this region. Such differences may also be a result of differences between DCX+ cell morphologies (multipolar/fusiform/round) and how their characteristics (size, number of processes, intensity of staining, etc.) contribute to overall %DCX+ coverage within a particular field of view.

Surprisingly, almost exactly opposite patterns were observed in HP2 (Figure 17; HP2 panel, second from top): there was no seasonal variation in %DCX+ coverage, except for in birds captured in spring (March-April), who showed increased coverage in the caudal portion of HP2 compared to rostral HP. It may be that the overall rostro-caudal differences in neurogenesis in the hippocampus are driven only by the HP1 subregion (dorsolateral), and that the change in coverage in caudal HP2 is a by-product of increased proliferation in spring in the nearby subventricular zone (the site of initial proliferation for all new neurons in the brain) to support increased recruitment to vocal control structures HVC and RA, both of which are located in the caudal portion of the brain (e.g., Hoshooley et al., 2005); spring peaks in HVC and RA neurogenesis in spring has been well-documented in many songbird species (reviewed in Brenowitz and Larson, 2015). While I have not yet examined neurogenesis in vocal control regions to confirm if this is indeed the case, if the observed difference in caudal HP2 is indeed an artifact of proliferation in the nearby caudal SVZ, the lack of an overall rostro-caudal difference in the spring in both HP1 and HP2 subregions may be a result of decreased reliance on spatial memory when food sources become more abundant in spring; these patterns may only occur in the fall and winter when birds are storing and retrieving food.

Seasonal patterns of %DCX+ coverage along the rostro-caudal axis differed between HP1 and the nearby control region HA, where no rostro-caudal differences were found across all seasons except in December, where there was markedly greater %DCX+ coverage in the caudal portion of the brain (Figure 17; HA panel, second from bottom). A recent study by Mehlhorn et al. (2022) showed a similar rostro-caudal pattern of DCX-ir (albeit not seasonally driven) in the HA of pigeons; with more immature neurons in the caudal HA compared to the rostral section. Overall, as described above, seasonal patterns

of neurogenesis in HA warrant further investigation in black-capped chickadees, particularly if HA is also implicated in some degree of spatial coding.

Lastly, there was a consistent difference along the rostro-caudal axis in the lateral nidopallium, with no apparent seasonal influence. Across all seasons, there was more %DCX+ coverage in the rostral LNP than in the caudal LNP (Figure 17; LNP panel, bottom). As in HA, there is little prior work on rostro-caudal patterns of DCX-ir in LNP, however Mehlhorn et al. (2022) showed similar trends in the overall nidopallium of pigeons: showing significantly more new neurons (DCX+ and BrdU colocalized with GFAP) in the rostral nidopallium relative to the caudal nidopallium.

2.4.5 CONCLUSIONS

Here, I show, for the first time, evidence for sex-specific seasonal variation in hippocampal plasticity in black-capped chickadees. Given that this data deviates from the bulk of previous studies on this topic, replication of these data using a similarly large (but preferably larger) sample of wild male and female black-capped chickadees is critical. Further examination of how these female-biased seasonal differences in the brain correspond to differences in hippocampus-dependent spatial behaviour in chickadees is also warranted; however it is imperative that such future studies take great care to ensure high levels of ecological validity when examining the behaviours of wild food-storing birds and to mitigate potentially confounding effects of captivity.

2.5 FIGURES

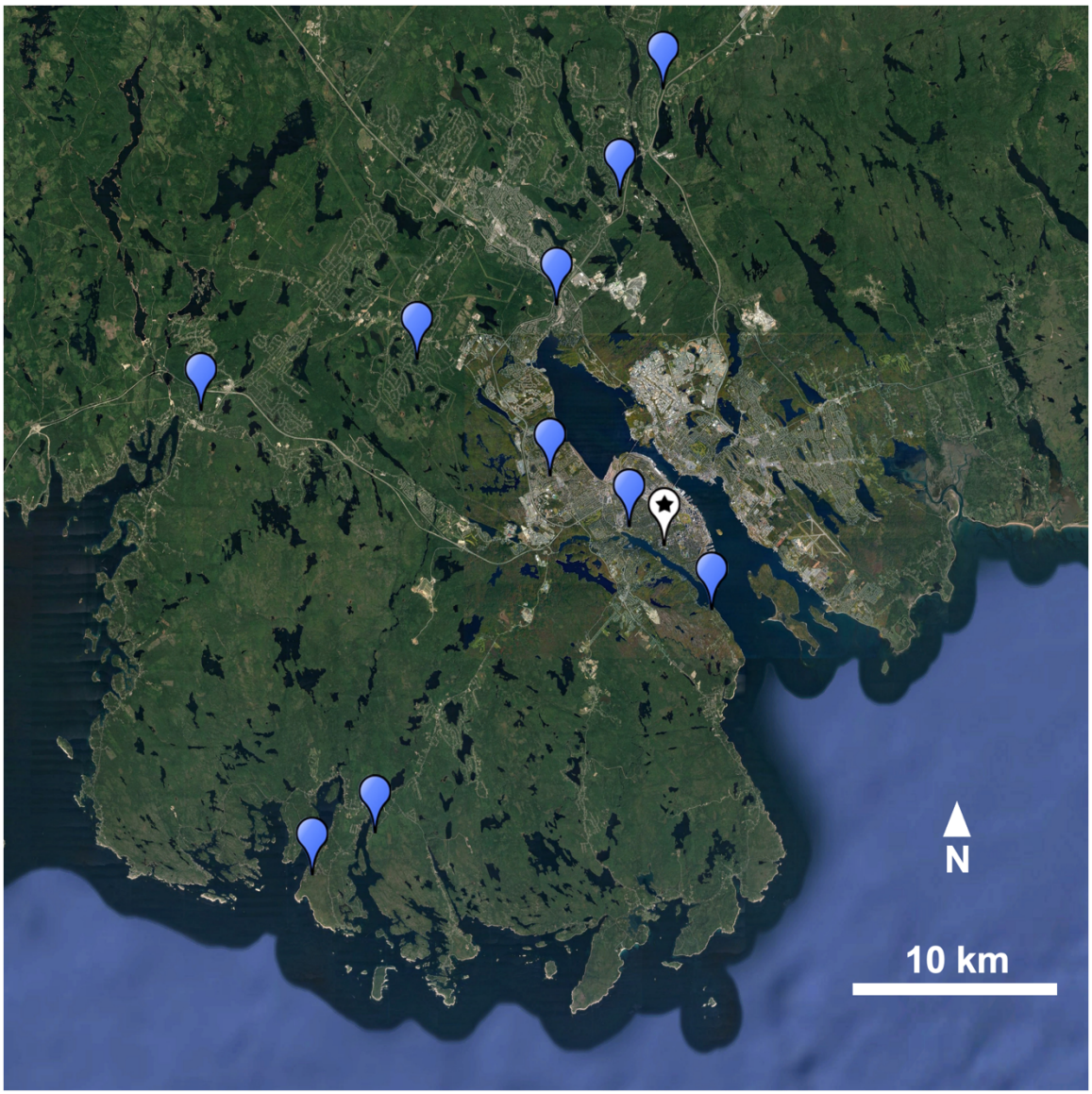

Figure 1. Map of Halifax, Nova Scotia and surrounding areas, indicating the ten sites (blue markers) where black-capped chickadees were captured for use in this study. White star indicates location of the laboratory (Life Sciences Centre, Dalhousie University, Halifax, NS). Created using Google Earth Pro.

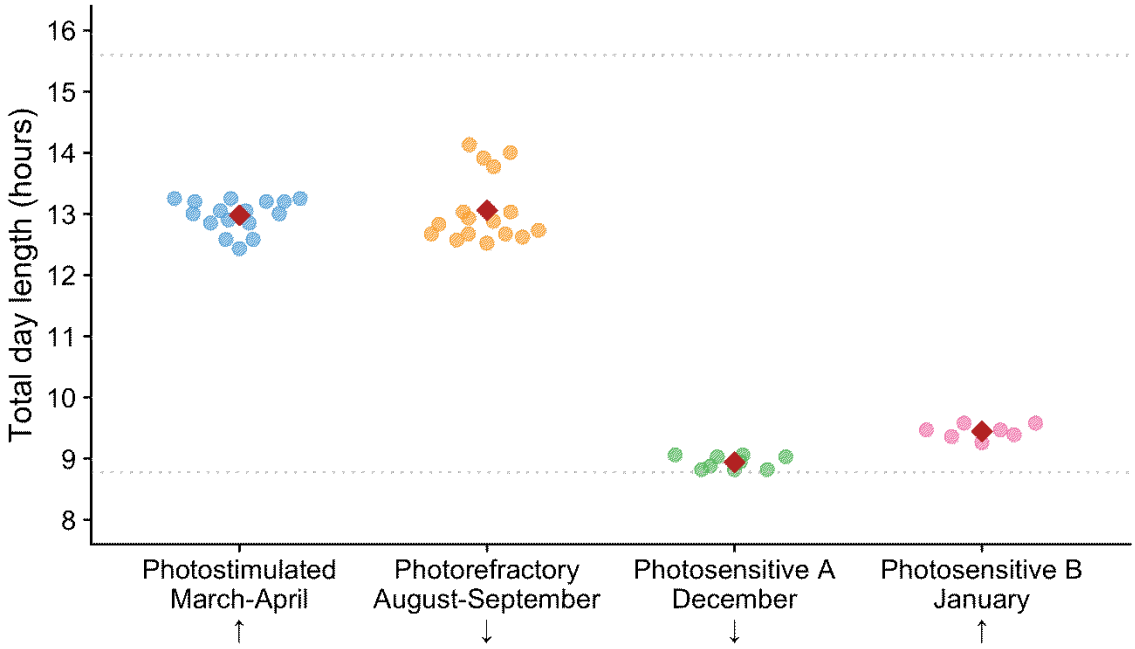

Figure 2. Day length associated with each photoperiodic condition. Individual data points represent day lengths for day of capture for each subject, calculated using the approximate coordinates for each capture site. Red diamond indicates mean. Arrows below labels on x-axis indicate increasing (↑) or decreasing (↓) day length. Dashed lines indicate day lengths associated with shortest (December 21, 2021, 8.78 hours) and longest (June 21, 2021, 15.6 hours) of the year 2021 (and overall capture period), as calculated by the NRC Sunrise/Sunset Calculator for Halifax, Nova Scotia.

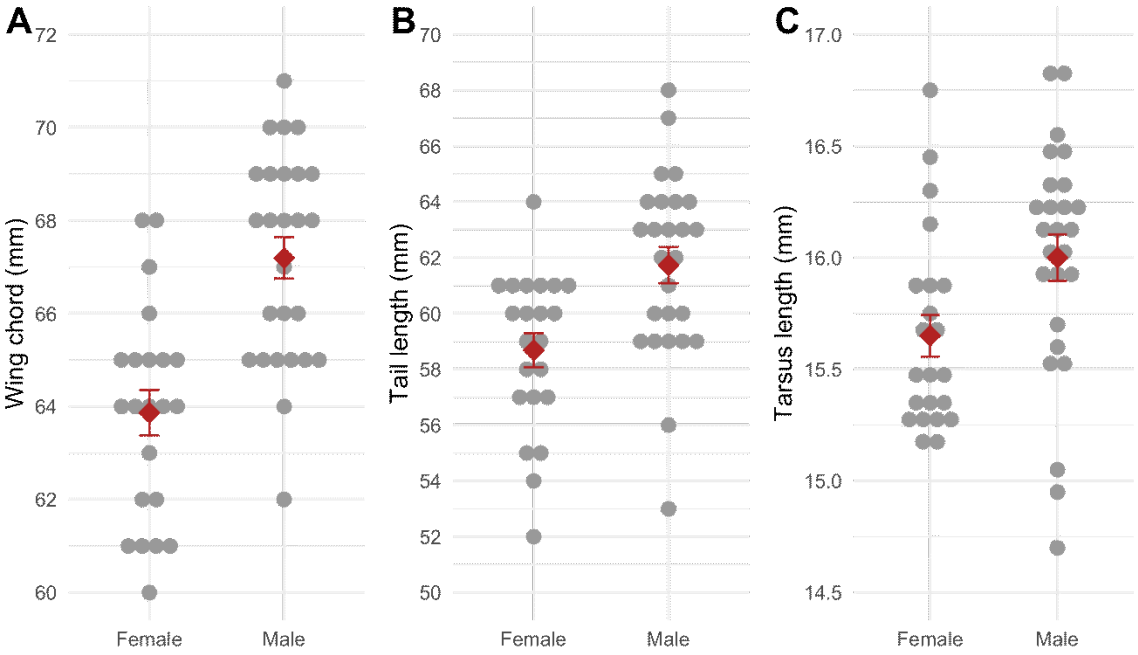

Figure 3. Morphological data − wing chord (A), tail length (B), tarsus length (C) − for all birds captured in this study, separated by sex. Red diamonds and error bars indicate mean ± SE.

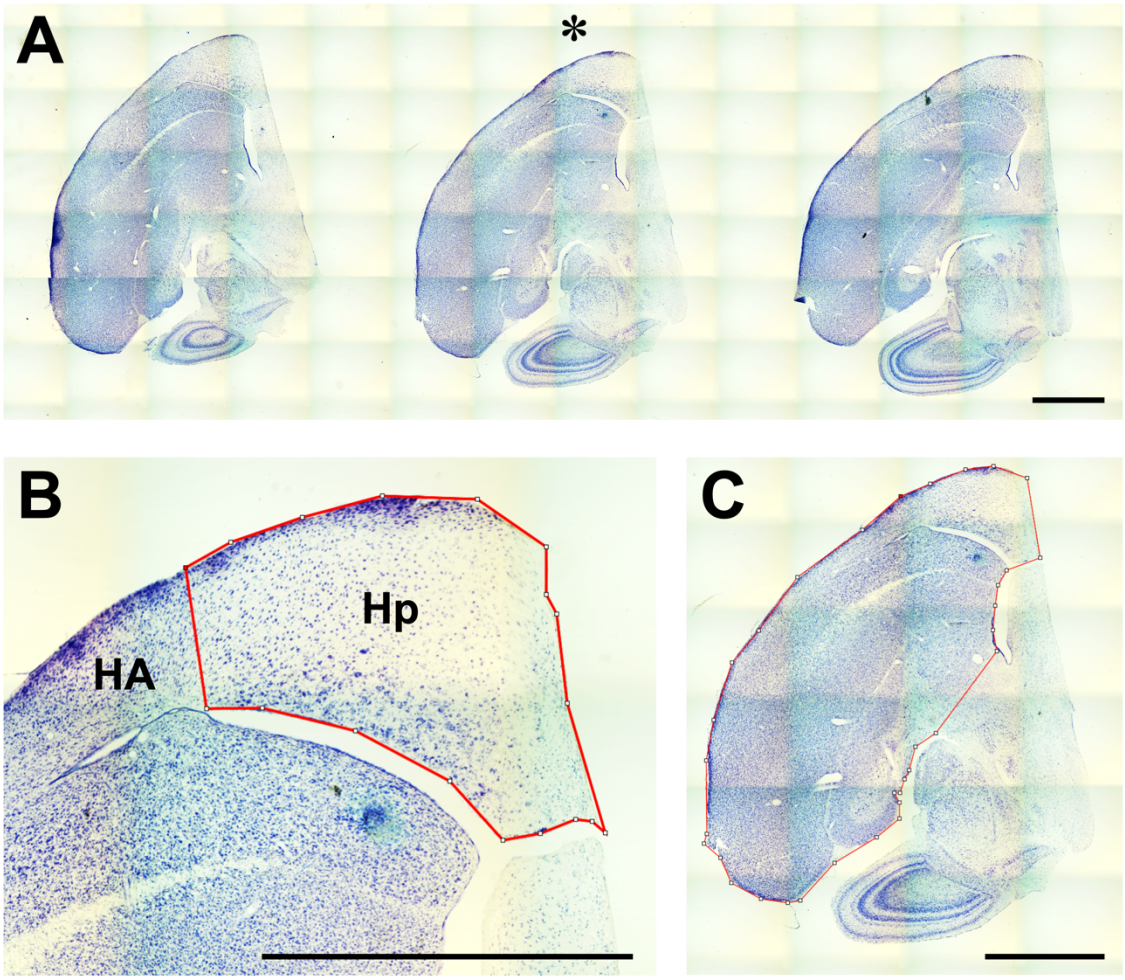

Figure 4. Example photomicrographs of cresyl violet-stained brain tissue from one subject (panel A, middle section, ✻). Panel A shows part of a whole-slide image, consisting of individual photomicrographs (captured using 4× objective) stitched together using the Multiple Image Alignment feature in Olympus cellSens Dimension. Panels B and C show traced areas of the hippocampus (B) and the telencephalon (C) used to reconstruct the volume of these regions. The cytoarchitectural border between the hippocampus (Hp) and the adjacent region hyperpallium apicale (HA; shown in Panel B), described initially by Sherry et al. (1989) determined the lateral boundary of the hippocampus. All scale bars (bottom-right of each image) indicate 2 mm.

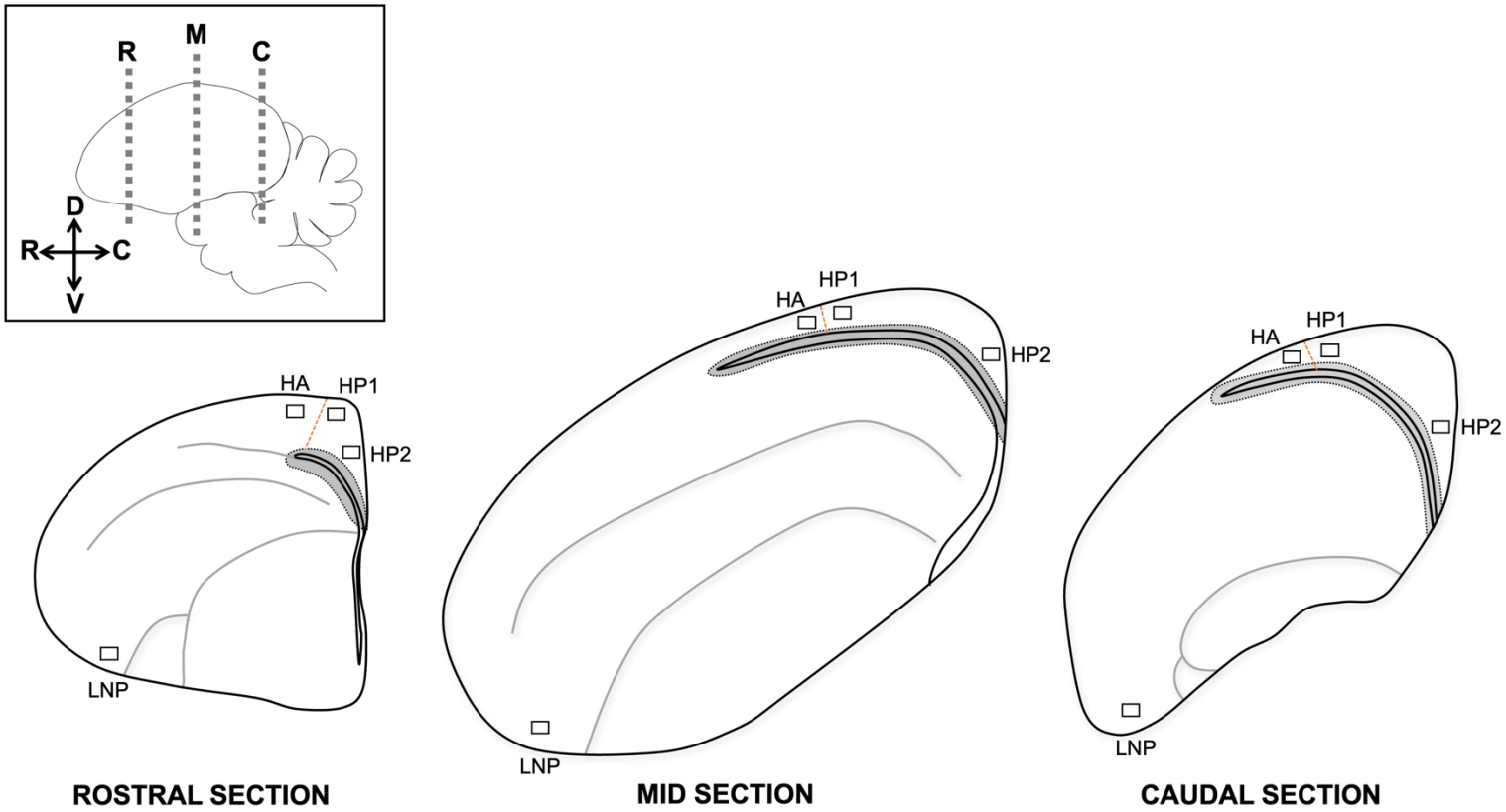

Figure 5. Line diagram of one hemisphere of the black-capped chickadee telencephalon (coronal section) across three representative planes of section (rostral, mid, caudal). In each section, I captured four images to quantify neurogenesis using doublecortin (DCX) immunohistochemistry: two in the hippocampus (HP1 and HP2), one in the nearby hyperpallium apicale (HA; control), and one in the lateral nidopallium (LNP; control). Dashed line (orange) in each section represents the approximate location of the cytoarchitectural boundary between the hippocampus and HA. Stippled line (shaded in grey) represents the subventricular zone. Box (upper-left corner) indicates location of rostral (R), mid (M), caudal (C) sections in a sagittal view of the brain; sagittal line drawing adapted from Parks et al. (2021).

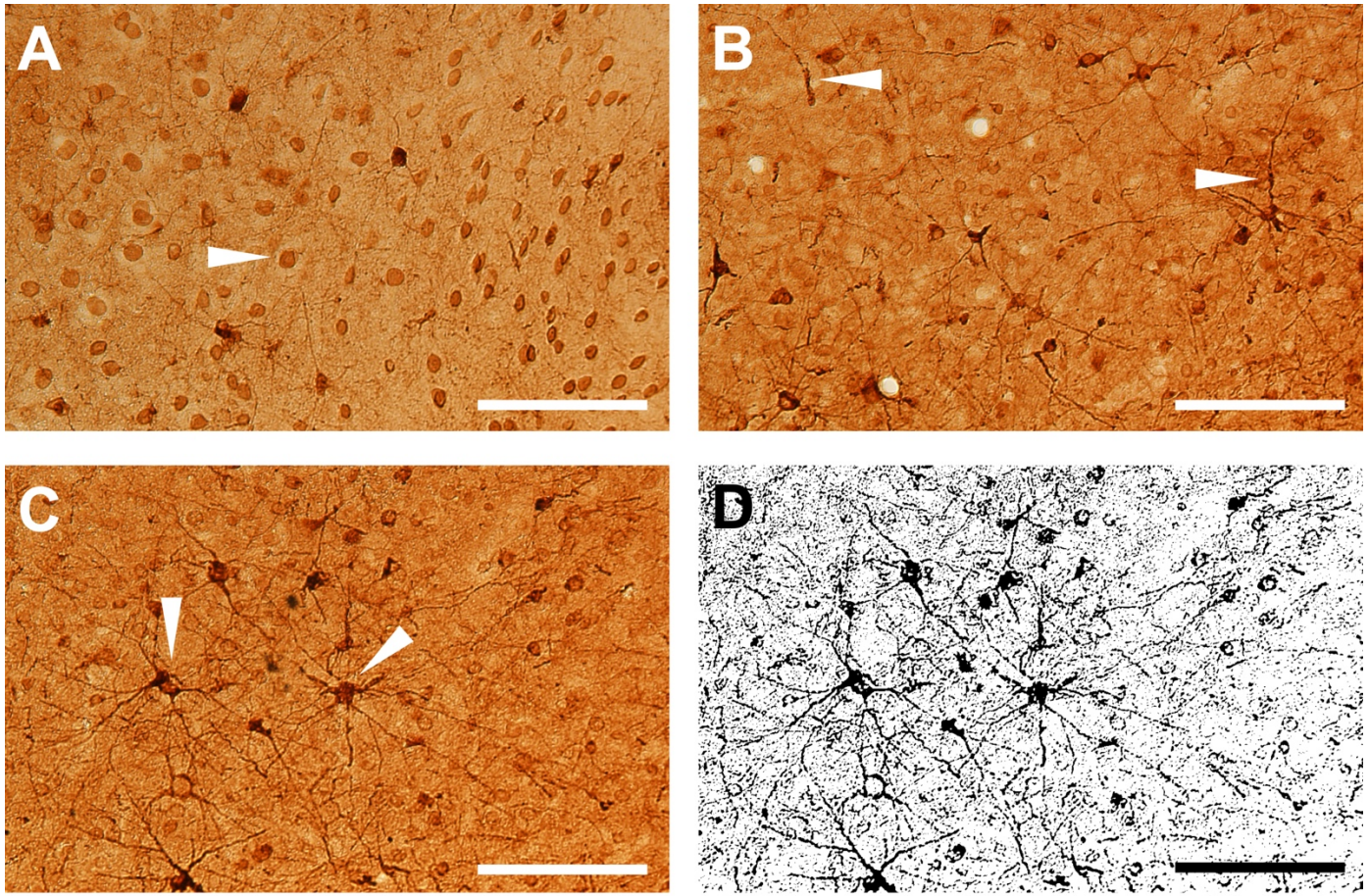

Figure 6. Example photomicrographs of immature neurons stained for doublecortin (DCX; visualized with 3,3'-diaminobenzidine) in the hippocampus (A) lateral nidopallium (B) and hyperpallium apicale (C-D). White arrowheads in each panel indicate a different DCX+ cell type I quantified in this study: round cells (A), fusiform cells (B), and multipolar cells (C). Panel D shows an example of the automated thresholding plug-in in FIJI used to quantify %DCX+ coverage seen in Panel C. All scale bars (bottom right corner) represent 100 μm.

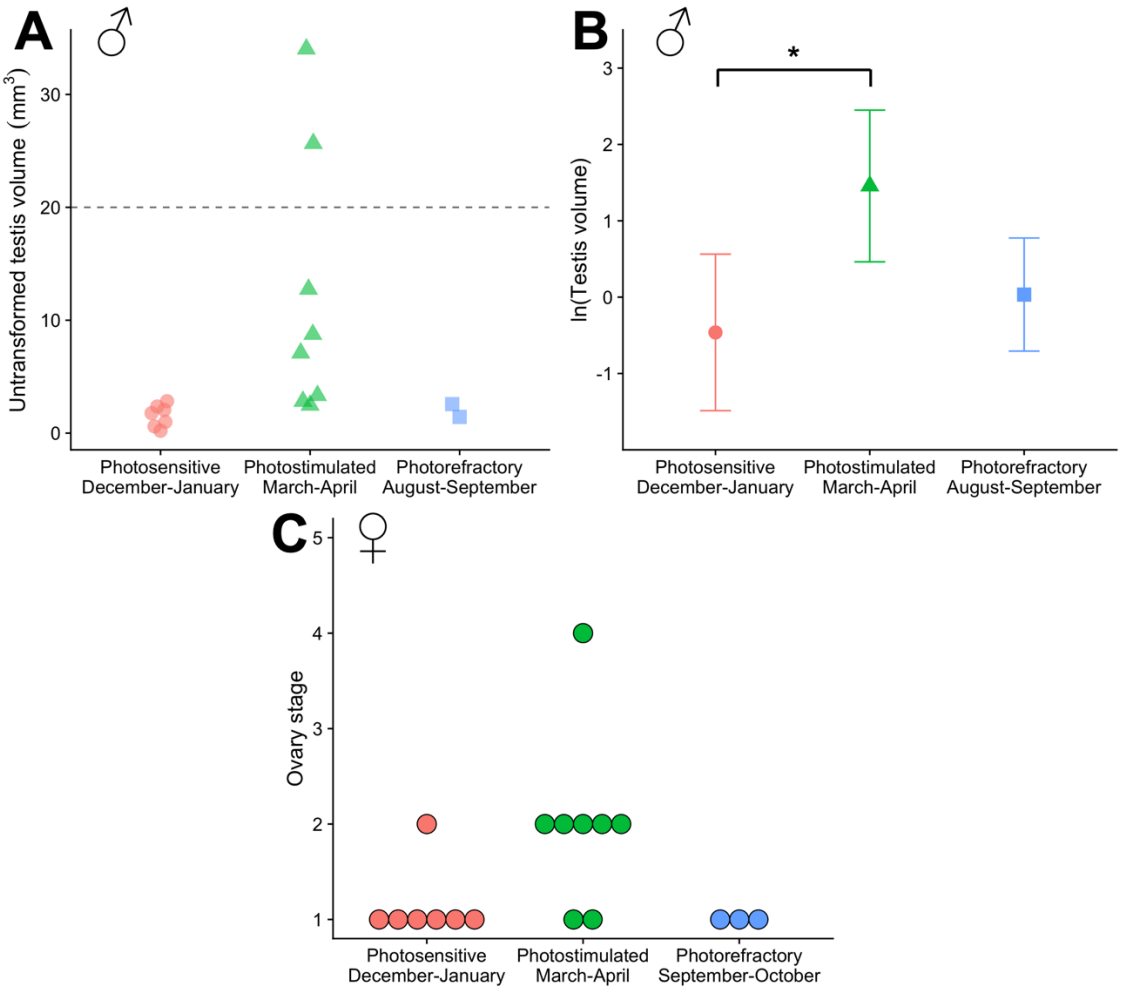

Figure 7. Gonad data from male (A-B) and female (C) black-capped chickadees captured for this study. Panel A shows the raw untransformed testis volume; data points represent individual birds. Dashed line indicates the 20 mm^3 threshold for photostimulation described by Phillmore et al. (2006). Panel B show the overall differences in testis volume (natural-log-transformed) across three photoperiodic conditions; points and error bars indicate mean ± 95% CI; * indicates $p < .05$. Panel C shows stage of ovary development in females; data points represent individual birds.

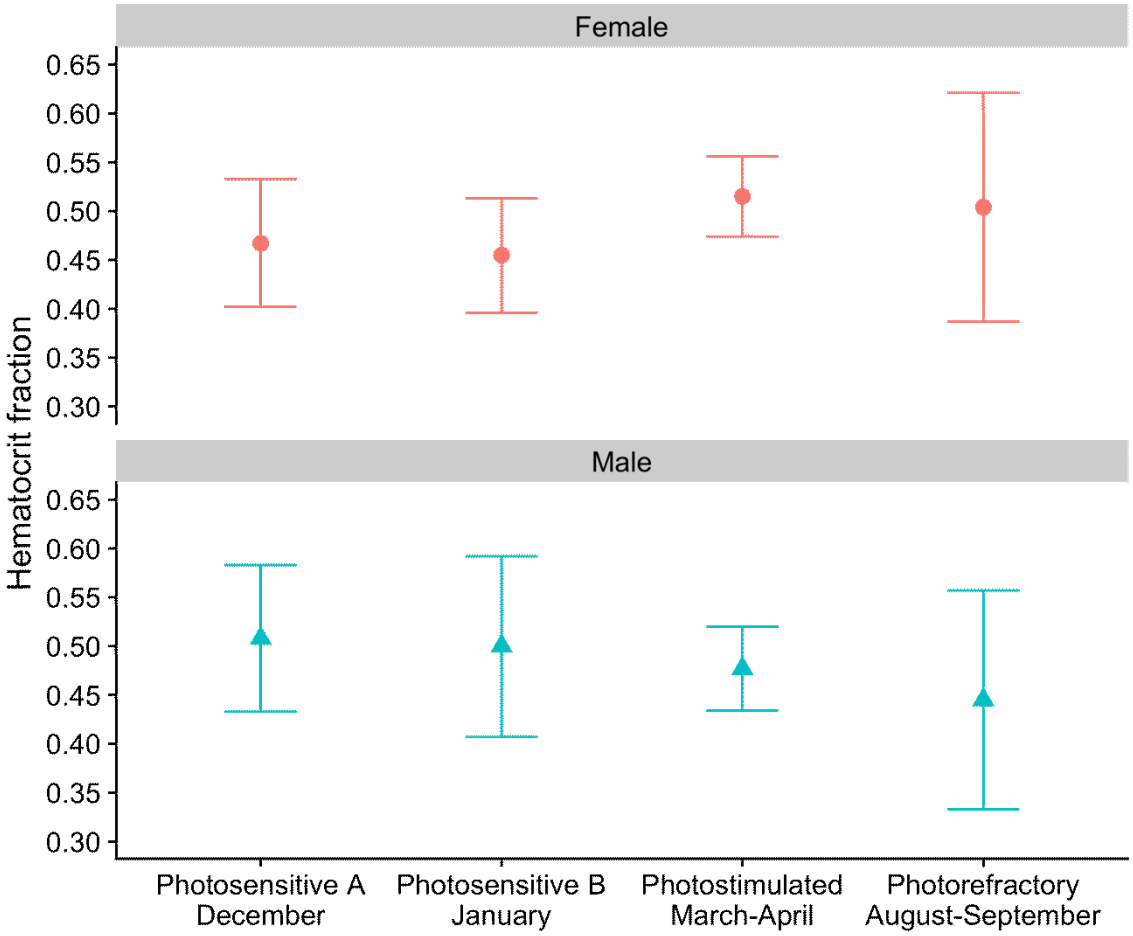

Figure 8. Seasonal variation in hematocrit fraction in female (red circles) and male (blue triangles) black-capped chickadees. Points and error bars represent mean ± 95% CI.

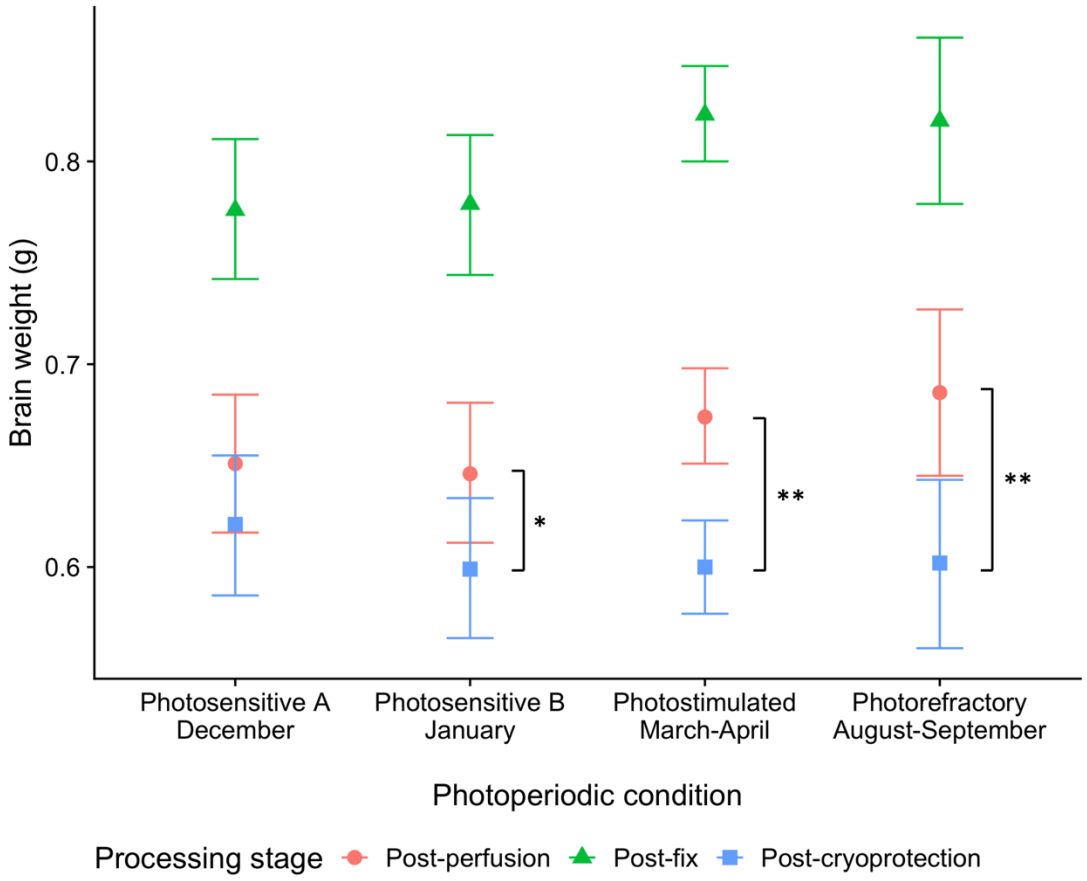

Figure 9. Seasonal variation in brain weights across three brain processing stages: post-perfusion (red circles), post-fix (green triangles) and post-cryoprotection (blue squares). Points and error bars represent mean ± 95% CI. * indicates $p < .05$; ** indicates $p < .001$.

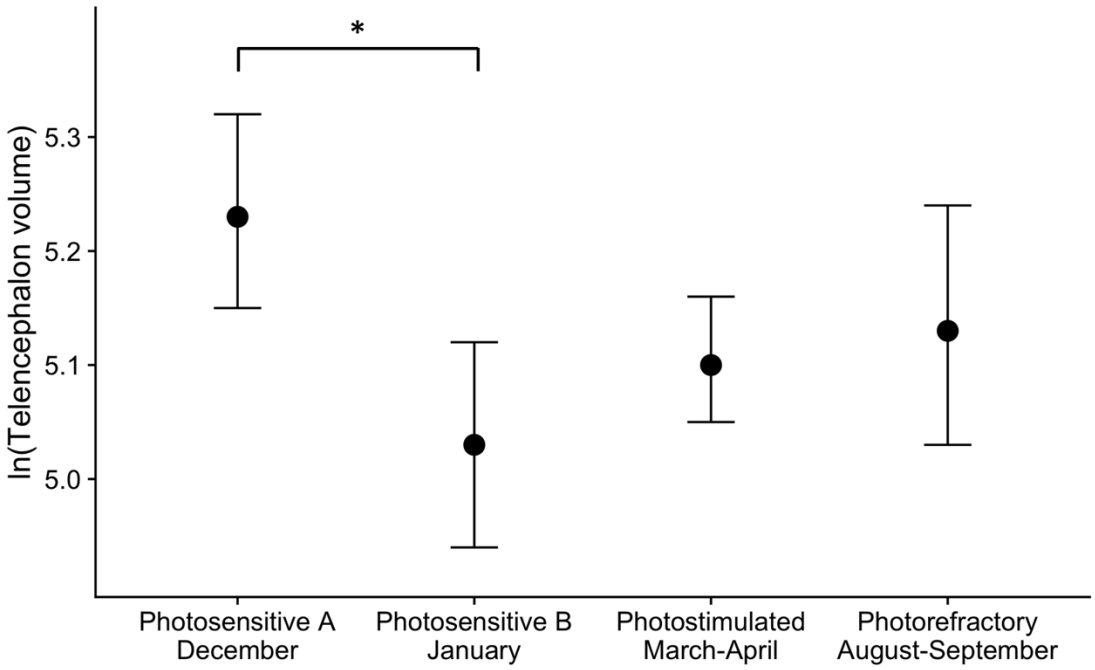

Figure 10. Seasonal variation in telencephalon volume (natural log transformed), combined between sexes. Points and error bars represent mean ± 95% CI. * indicates $p <$ .05.

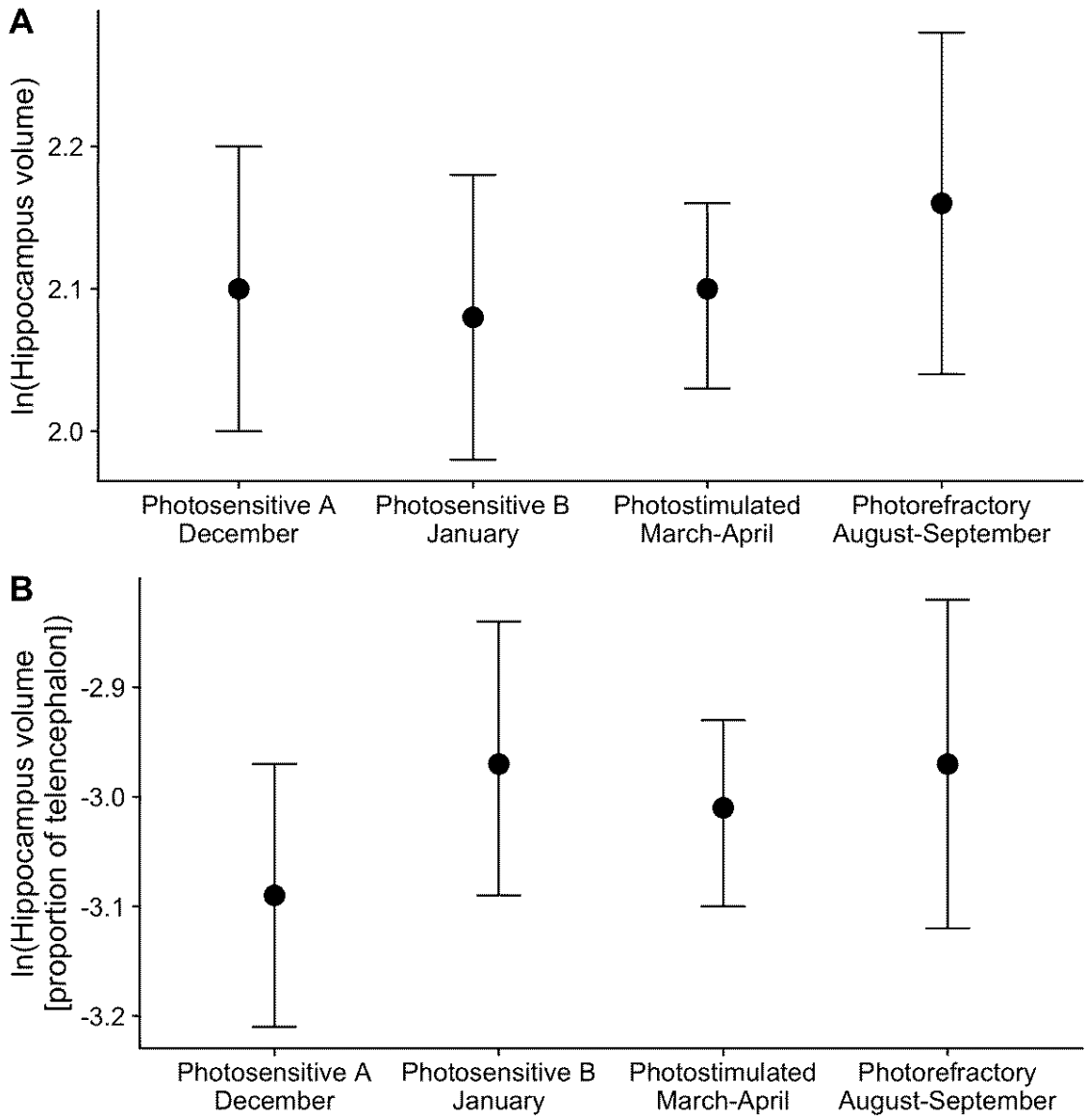

Figure 11. Seasonal variation in overall hippocampal volume (A) and hippocampal volume as a proportion of the telencephalon (B). Points and error bars represent mean ±

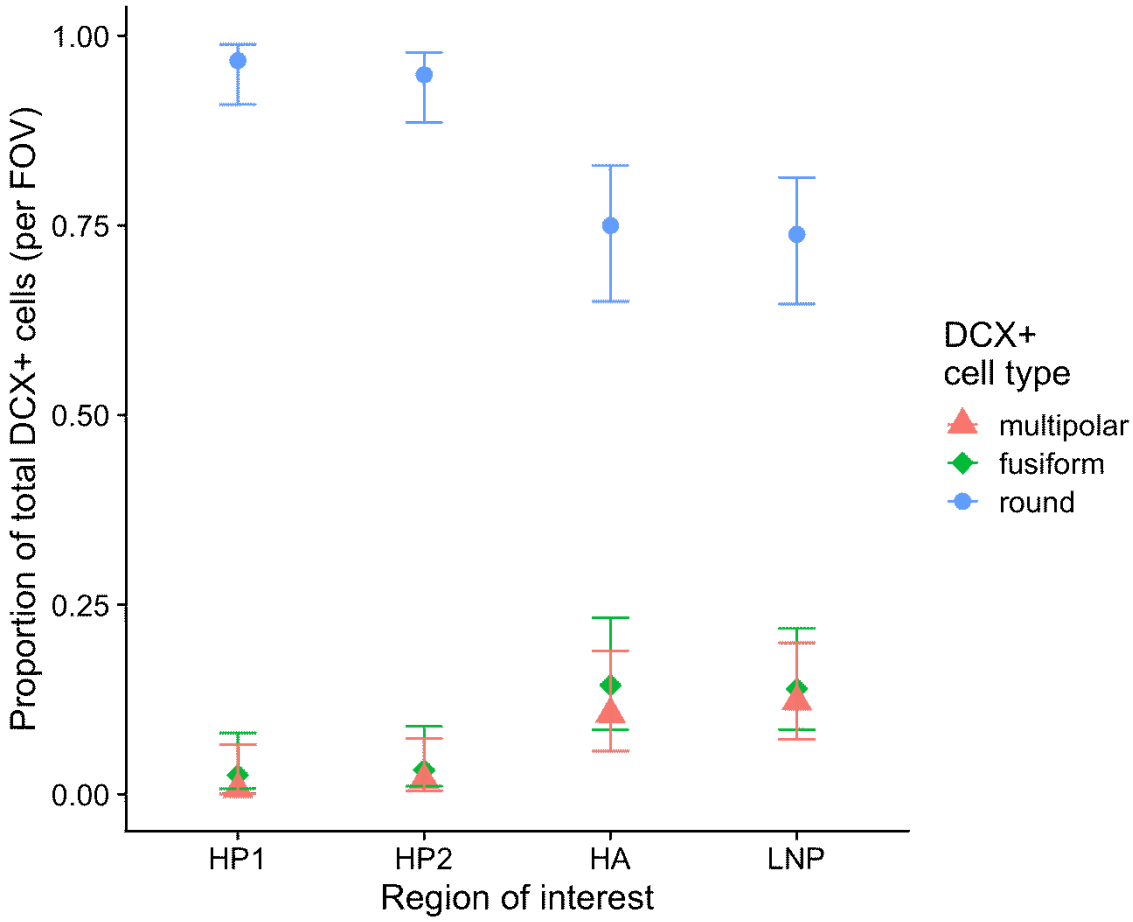

Figure 12. Brain region differences in the proportion of each DCX+ cell type – multipolar (red triangle), fusiform (green diamond), and round (blue circle) per total DCX+ cells per microscope field of view, across all four brain regions. Points and error bars represent mean ± 95% CI.

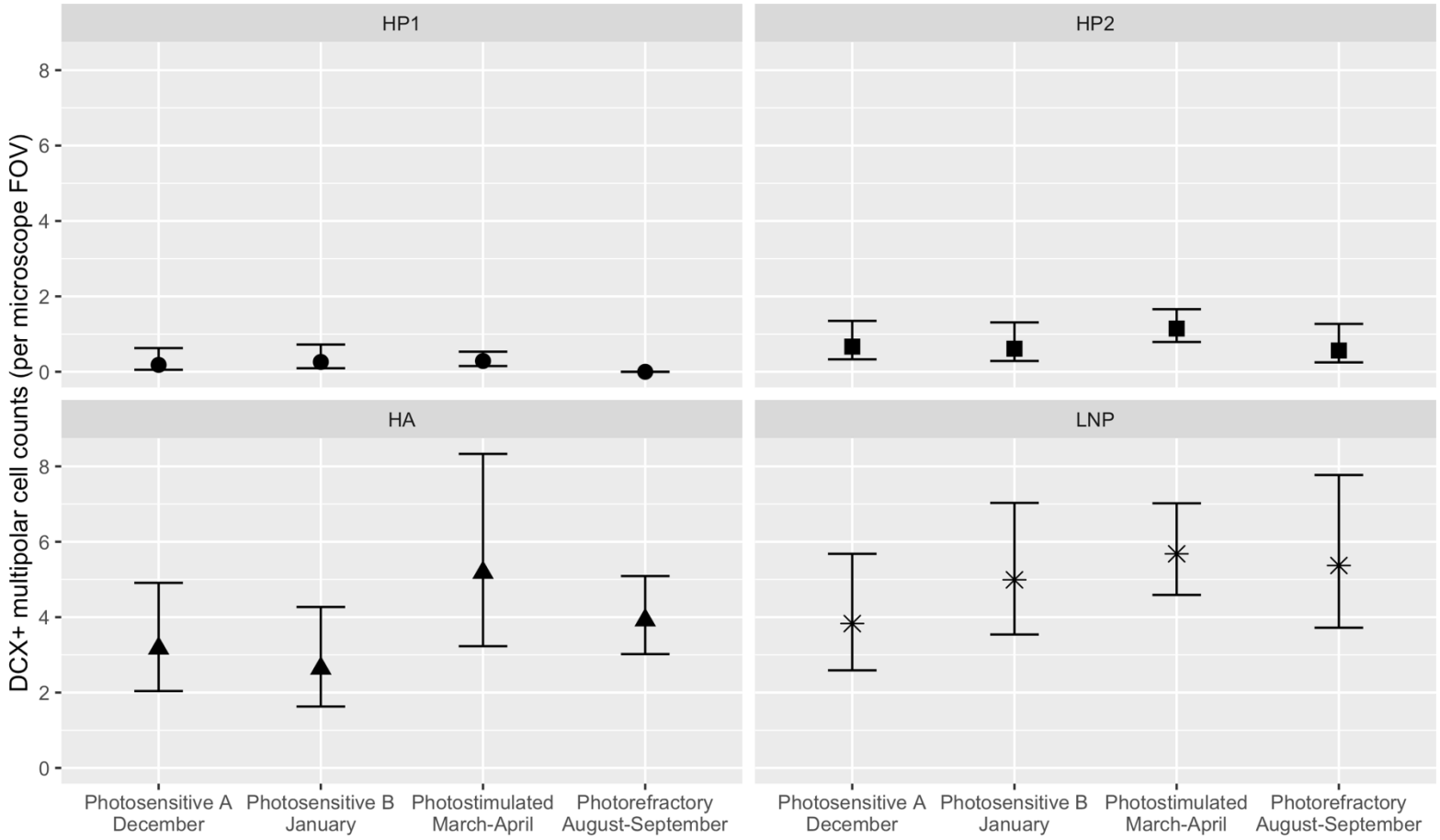

Figure 13. Seasonal variation in number of DCX+ multipolar cells across four brain regions of interest. Points and error bars represent mean ± 95% CI.

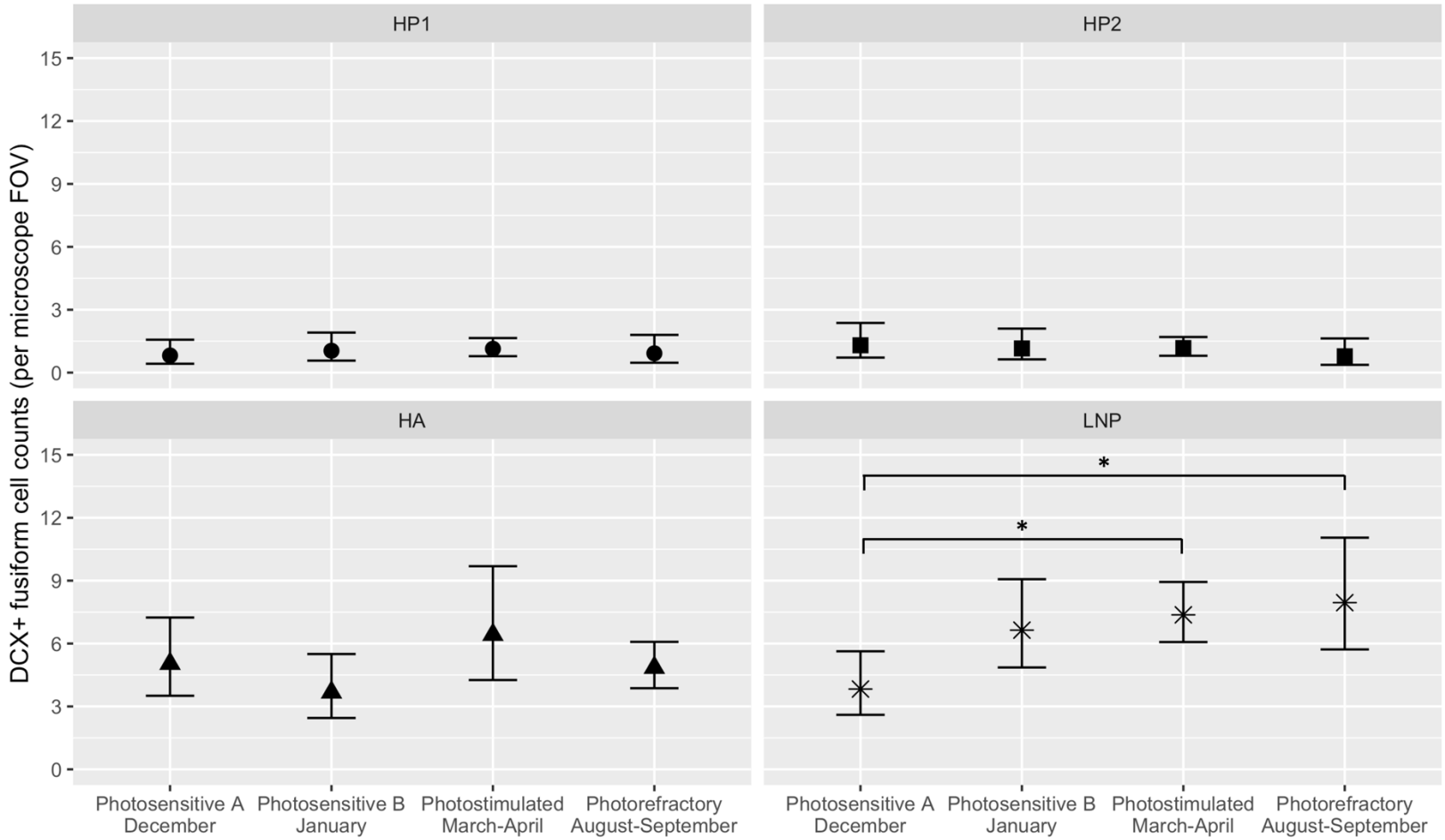

Figure 14. Seasonal variation in number of DCX+ fusiform cells across four brain regions of interest. Points and error bars represent mean ± 95% CI. * indicates $p < .05$.

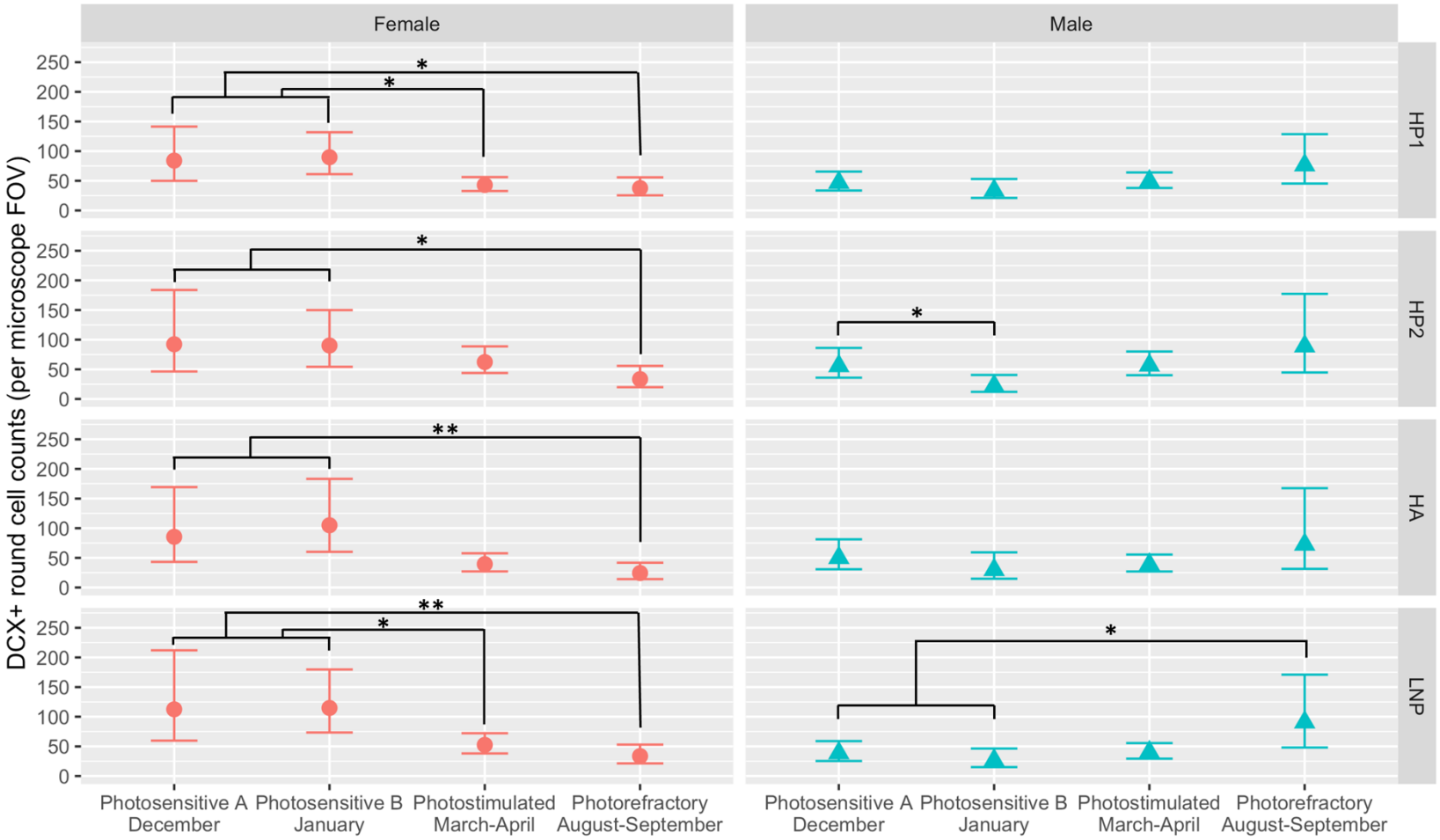

Figure 15. Seasonal variation in number of DCX+ round cells across four brain regions of interest, separated by sex: females (red circles; left) and males (blue triangles; right). Points and error bars represent mean ± 95% CI. * indicates $p < .05$; ** indicates $p < .01$.

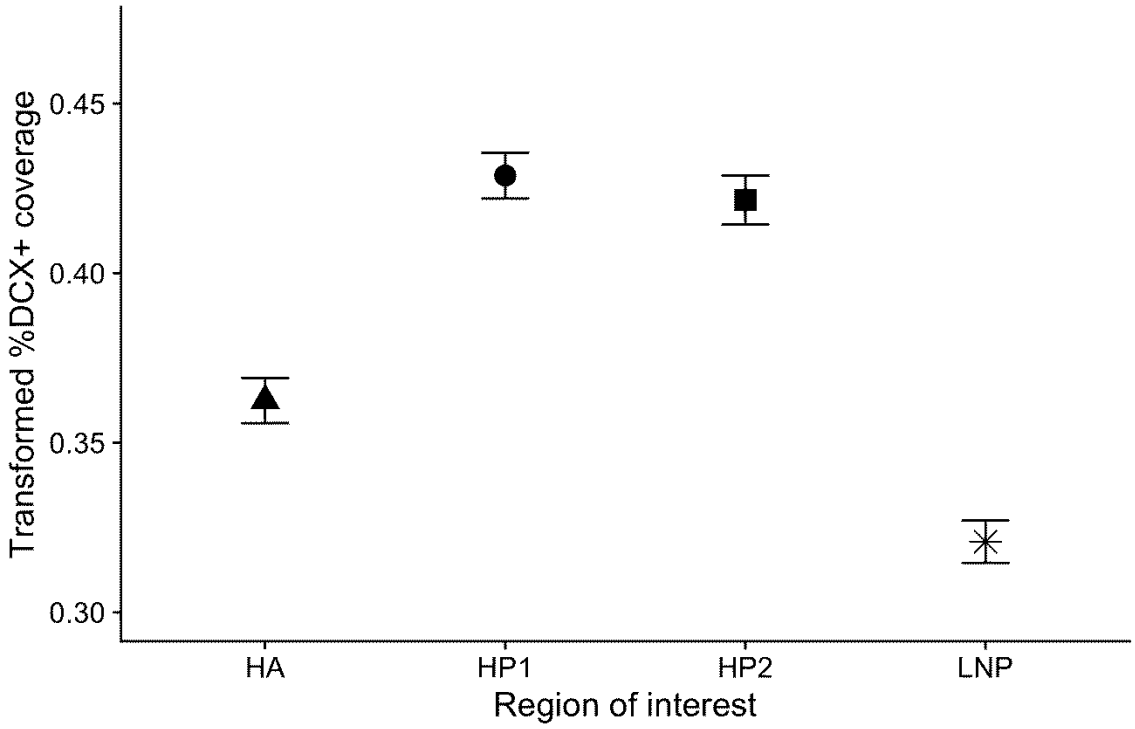

Figure 16. %DCX+ coverage across the four brain regions of interest (arcsine-transformed). Pairwise comparisons indicated significant differences in %DCX+ coverage except for between both hippocampal subregions (HP1 and HP2). Points and error bars represent mean ± 95% CI.

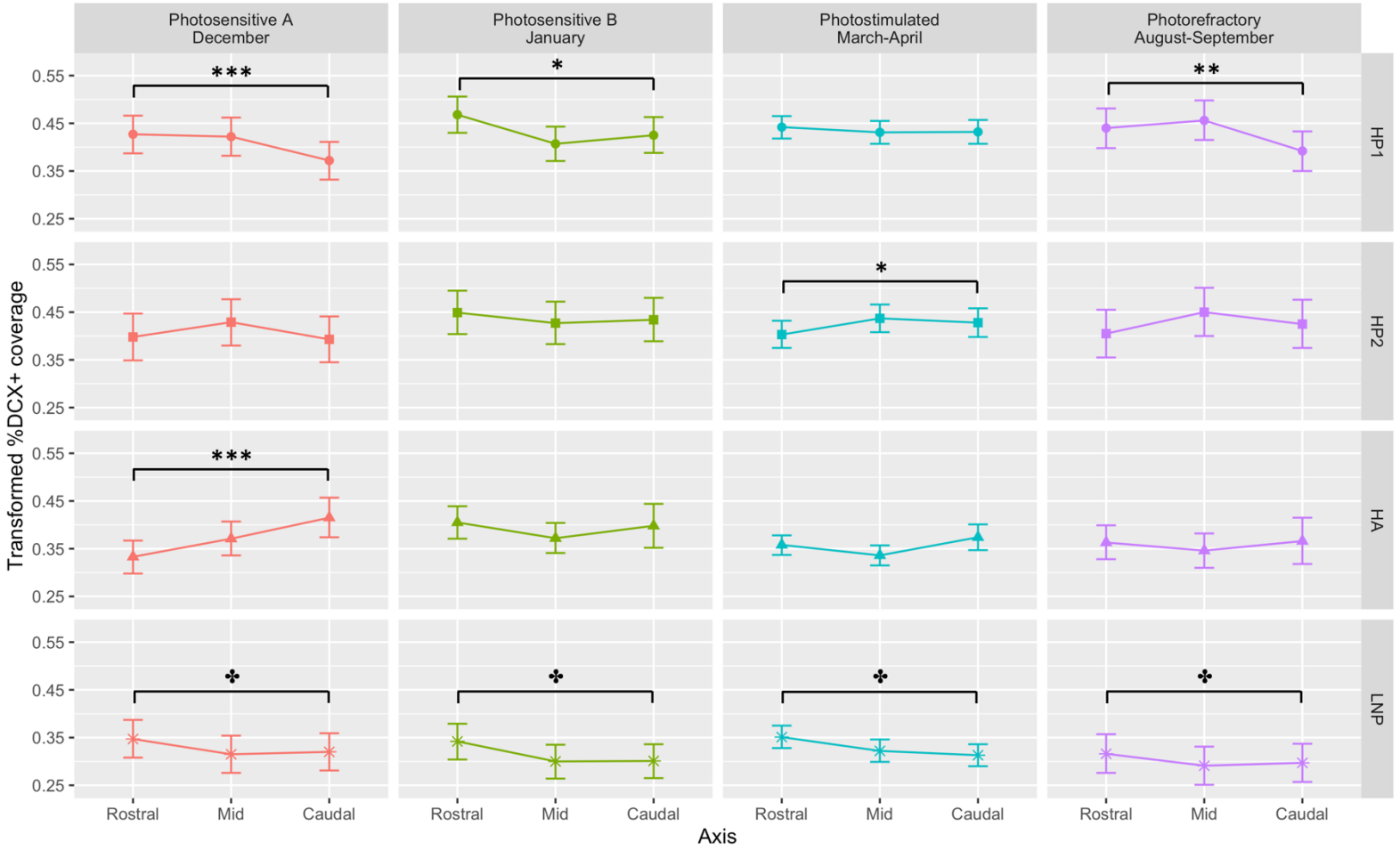

Figure 17. Seasonal variation in %DCX+ coverage along the rostro-caudal axis in all four brain regions of interest. * indicates $p < .05$; ** indicates $p < .01$; *** indicates $p < .001$. In the lateral nidopallium (LNP; bottom row), I detected a main effect of axis across all seasons; ✤ indicates Bonferroni-adjusted $p < .05$. Points and error bars represent mean ± 95% CI.

2.6 TABLES

Table 1

Overview of groups, capture dates, natural photoperiod associated with capture dates used in this study.

Predicted photoperiodic condition	Capture dates	Mean (range) day length (in hours)	Increasing or decreasing day length	Birds captured
Photostimulated	25 March-10 April 2021	12.98 (12.43-13.25)	Increasing	16
Photorefractory	13 August-15 September 2021	13.06 (12.52-14.13)	Decreasing	16
Photosensitive A	1-18 December 2021	8.94 (8.82-9.06)	Decreasing	9
Photosensitive B	17-26 January 2022	9.44 (9.26-9.58)	Increasing	7

Table 2A

Breakdown of all subjects captured in this study, separated by age and sex

Condition	Males captured	Females captured	Total
Photostimulated	8 adults	8 adults	16 adults
Photorefractory	2 adults 8 juveniles	3 adults 3 juveniles	5 adults 11 juveniles
Photosensitive A	5 adults	3 adults 1 juvenile	8 adults 1 juvenile
Photosensitive B	3 adults	4 adults	7 adults
Total	26	22	48

Table 2B

Breakdown of subjects included in analyses, separated by sex

Condition	Adult males included in analyses	Adult females included in analyses	Total
Photostimulated	8	8	16
Photorefractory	2	3	5
Photosensitive A	5	3	8
Photosensitive B	3	4	7
Total	18	18	36

Table 3

Ordinal scale (first used in studies of black-capped chickadees by MacDougall-Shackleton et al., 2003) used to describe stage of ovary development in the subjects in this study.

Ovary stage	Description
1	Smooth, no visible follicular development
2	Slightly granular appearance
3	Small follicles apparent, but no follicular hierarchy
4	Obvious follicles with clear hierarchy
5	Large, yolky follicles

CHAPTER 3: GENERAL DISCUSSION

The overall objective of this study was to characterize seasonal patterns of neural plasticity in a wild population of male and female black-capped chickadees (*Poecile atricapillus*), a non-migratory species which relies on hippocampus-dependent spatial memory to store and retrieve food in order to survive through periods of the year (generally fall and winter) when conditions are harsh and traditional food sources are scarce. I captured birds at four time points (March-April, August-September, December, and January) over the course of a year. Instead of keeping birds in captivity, as in some prior studies of seasonal neural plasticity in this species (e.g., Barnea and Nottebohm, 1994; Hoshooley and Sherry, 2007; Hoshooley et al., 2007), birds in this study were sacrificed shortly (ca. 1 hour) after capture. I quantified two measures of hippocampal neural plasticity – volume and neurogenesis, as well as other neural and physiological measures including overall brain weight and hematocrit. I found some evidence of seasonal variation in hippocampal plasticity, and interestingly, this seasonal variation was largely restricted to female black-capped chickadees, who, unlike males, showed a marked peak in hippocampal neurogenesis in January.

3.1 REGION-SPECIFIC DIFFERENCES IN DOUBLECORTIN (DCX) IMMUNOREACTIVITY IN BLACK-CAPPED CHICKADEES

In their study of European starlings, Hall et al. (2014a) were the first to describe a hippocampus-specific type of DCX+ cells, referring to these cells as “hippocampal spherical”, as they displayed a similar cellular phenotype to “spherical” cells (another term used to describe the cell type I refer to here as multipolar). Further, they noted that “hippocampal spherical” DCX+ cells were the only DCX+ cellular phenotype observed in the starling hippocampus (Hall et al., 2014a), however no data is provided in support of this assertion, and it is unclear whether these “hippocampal spherical” cells were observed in any other their other ROIs (striatum, nidopallium). Similarly, Taufique et al. (2018) quantified DCX+ cells in the hippocampus of Indian house crows using the same three categories (multipolar, fusiform, and hippocampal spherical) as in Hall et al. (2014a), showing that these “hippocampal spherical” cells were also the predominant cell type in the hippocampus of this species. However, as in Hall et al. (2014a), Taufique et

al. (2018) only examined spherical cells in the hippocampus, and, as in Hall et al. (2014a), this study was not clear as to whether the lack of reporting of "spherical cells" in their other ROIs (subregions of the nidopallium) meant this "spherical" cell type was absent from their other ROIs or were simply combined with another cell type (i.e., multipolar) for comparative purposes. Lynch (2018) used DCX-ir to quantify subregional differences in hippocampal neurogenesis in brown-headed cowbirds and also noted that the round morphology was the most abundant cell type across the hippocampus and in all hippocampal subregions, although both multipolar and fusiform cells were also present.

Here, I add to Hall et al. (2014a), Taufique et al. (2018) and Lynch (2018) by showing that round cells are indeed the predominant DCX+ cell type in the hippocampus of black-capped chickadees, in significantly higher proportions compared to control regions HA and LNP (Figure 12). Further, seasonal hippocampal plasticity in female black-capped chickadees was indeed restricted only to round cells (Figure 15): there was no evidence of seasonal change in either of the other two, more well-characterized DCX+ cellular phenotypes (multipolar and fusiform) in the hippocampus (see Figures 13-14). These two lines of evidence, taken together, suggest that round cells in the hippocampus may play a role in supporting spatial behaviours in wild birds.

Interestingly, in their initial study describing patterns of DCX-ir across the songbird brain, Boseret et al. (2007) describe four distinct DCX+ cell types in the canary brain: (1) "densely stained round multipolar cells"; (2) "fusiform elongated cells"; (3) "weakly stained cells with few immunostained processes"; and (4) "weakly stained cells […] associated with small immunoreactive dots reminiscent of punctate structures" (p. 145; with image examples). Indeed, the images of the third cell type they described ("round with few immunostained processes") very much resemble the images of the "round" or "hippocampal spherical" morphology shown in this study (Figure 6), as well as those by Hall et al. (2014a, see image on p. 878) and Lynch (2018, see image on p. 49). However, Boseret et al. (2007), and later Balthazart et al. (2008) dismiss the latter two cell types as being "obviously not associated with adult neurogenesis" (Balthazart et al., 2008; p. 806), suggesting that these cells, while expressing DCX, are simply evidence of non-neurogenic "changes in the dendritic tree" (Boseret et al., 2007; p. 152; see also Balthazart and Ball 2014b, pp. 4111-4112).

By these original definitions and (speculative) functional descriptions described first in Boseret et al. (2007) and later by Balthazart et al. (2008), the "round" DCX+ cell type observed here in large proportions (≥ 95%; Figure 12) in the hippocampus of black-capped chickadees, as well as the seasonal fluctuations in the quantities of this specific cell type in the hippocampus (which are largely in line with other studies showing a similar pattern of seasonal fluctuation in hippocampal neurogenesis, e.g., Hoshooley et al., 2007; albeit not in females) are not in fact immature neurons and have nothing to do with the process of adult neurogenesis. This scenario, while theoretically possible, is unlikely. A more plausible explanation is that species of study is an important consideration in the study of adult neurogenesis in birds, particularly comparing species that are, and are not, spatial specialists.

Relative to the vast body of work related to other neural structures and circuits in the songbird brain (most notably the vocal control system), the songbird hippocampus still remains relatively understudied. Perhaps less focus was given to the hippocampus during data analysis in these original studies and as a consequence, less overall data regarding fine-scale patterns of DCX-ir in this region were not available. Indeed, the hippocampus is only considered briefly in these initial DCX studies in canaries (a non-spatial-specialist). The only acknowledgement of the hippocampus in Boseret et al. (2007) is: "the hippocampus and the area parahippocampalis […] contained a high number of DCX+ cells throughout their rostro-caudal extent" (p. 148). It may be that canaries, domesticated songbird species who often spend the entirety of their lives in captivity, have relatively underdeveloped hippocampi; therefore the patterns of neurogenesis in this region may not be representative of those experienced by wild birds, particularly those with pronounced spatial capabilities (i.e., spatial specialists), including black-capped chickadees.

It is evident that further examination of the functional role of the various DCX+ cell types in adult neurogenesis in songbirds is needed, particularly with respect to round cells and the role they may play in hippocampus-dependent spatial behaviour. To fully understand the differences between DCX+ cell types, non-immunohistochemical methods, including more quantitative (e.g., electrophysiology) or molecular (e.g., single-cell sequencing) approaches is warranted to understand whether different morphologies

relate to different functions and/or features. Further, the lack of standardized nomenclature and/or classification system to describe and quantify DCX-ir in the songbird brain make comparing results across studies challenging; future studies must consider adapting such an approach to ensure consistency and reliability across the literature.

Indeed, comparatively little is known about the morphologies of hippocampal neurons (immature or mature) in songbirds relative to rodent models, for which entire databanks exist (e.g., "Hippocampome"; Wheeler et al., 2015; Sanchez-Aguilera et al., 2021) to describe and classify each of the 100+ distinct cell types characterized in the rodent hippocampus according to their morphologies, molecular markers, connectivities, *in vivo* recording characteristics, among others. The multipolar and fusiform morphologies described by Boseret et al. (2007) in the canary telencephalon are similar to the cellular morphologies observed and described in the first studies investigating DCX expression in the rodent brain (e.g., multipolar, Francis et al., 1999; fusiform, Gleeson et al., 1999). While smaller DCX-ir cells with no/few processes (similar morphologically to the round cells described here) were present in studies of non-hippocampal brain regions in rodents (e.g., guinea pigs, *Cavia porcellus*; Xiong et al. 2008; mice, *Mus musculus*; Batailler et al., 2014), there is some evidence that these cells also occur in the rodent hippocampus, and may be developmentally distinct from the other DCX+ cell morphologies.

In their study investigating the ontogeny of DCX+ expression (using multi-label immunofluorescence, co-localizing DCX with BrdU and NeuN), Brown et al. (2003) only describe two morphologies of DCX+ cells in the dentate gyrus of adult female Wistar rats, and suggest these cell types are developmentally distinct from one another: recently divided neuroblasts expressing DCX in the dentate gyrus appear to adopt a "round" morphology (described by Brown et al., 2003 as being "without defined processes" or "amorphous and short-branched", pp. 6-7) for up to 7 days post-mitosis. After ca. 7 days, most DCX+ cells develop processes at one end of the cell (akin to fusiform cells) which go on to span the granule cell layer and into the molecular layer of the dentate gyrus. Similarly, Shapiro et al. (2007) show that in the dentate gyrus of adult male Sprague-Dawley rats, DCX+ cells adopt a round morphology for the first ca. 24 hours post-

mitosis, however these cells develop the morphological characteristics of multipolar or fusiform cells within the first ca. 48 hours post-mitosis. These findings from rodent studies would suggest that perhaps the round cells observed in the avian hippocampus here may be early precursors to fusiform and multipolar DCX+ cells. However, whether this same developmental timeline (i.e., round cells only present within the first ca. 7 days, then differentiating into other morphologies) occurs in birds is unclear; a developmental study using multi-label immunofluorescence as in Brown et al. (2003) would be immensely beneficial to understanding the patterns of DCX expression in the songbird brain.

3.2 BEHAVIOURAL AND NEURAL PLASTICITY IN RESPONSE TO ENVIRONMENTAL CHANGE IN FOOD-STORING BIRDS

In contrast to several studies showing no differences in the hippocampus between male and female chickadees (e.g., Petersen and Sherry, 1996; Pravosudov and Clayton, 2002; Roth et al., 2010), the data reported here suggest seasonal patterns of hippocampal plasticity, particularly of hippocampal neurogenesis, do indeed vary between males and females: generally, males had consistent patterns of neurogenesis across the year while females showed evidence of seasonal change (Figure 15).

In a review of the neurobiology of spatial behaviour in birds and mammals, Lee et al. (1998) describe data derived from two published conference abstracts (Lee and Clayton, 1996; Lee et al., 1997) suggesting a female-biased sex difference in performance on a spatial learning task in Alaskan black-capped chickadees. In addition to chickadees purportedly performing better overall than non-food-storing white-crowned sparrows (*Zonotrichia leucophrys*) on the spatial task, they suggest their data also showed that female chickadees performed better than males; and most interestingly, this sex difference in performance was apparently not observed at all when testing black-capped chickadees in Colorado, a location with less harsh climate than Alaska, on the same task (Lee et al., 1998). These data, while largely uncorroborated, are, to my knowledge, the only evidence showing significant differences between males and female chickadees on any measure of behavioural or neural plasticity related to food-storing. Such behavioural

differences, if replicated, would lend credence to the findings reported here of seasonal hippocampal plasticity in females.

Since Lee et al. (1998), it has become well-established that spatial memory and hippocampal neural plasticity varies across populations of black-capped and mountain chickadees in different climates, along both long- (e.g., Pravosudov and Clayton, 2002; Roth and Pravosudov, 2009; Roth et al., 2011; Chancellor et al., 2011) and short-range (e.g., Freas et al., 2012; Branch et al. 2022) environmental gradients. For example, in a follow-up study to the design described by Lee et al. (1998), Pravosudov and Clayton (2002) showed birds captured in Alaska stored significantly more food, had significantly larger hippocampi (containing more neurons) and showed significantly better performance on spatial learning tasks than birds in Colorado; however males did not differ from females in hippocampal volume nor in number of hippocampal neurons.

Similarly, black-capped chickadees captured along a long-range environmental gradient (from Alaska to Kansas) were significantly different in terms of both hippocampal volume and hippocampal neuron number (Roth et al., 2011). However, data from male and female birds were pooled prior to testing for the effect of study site (i.e., latitude), as they did not detect an overall sex difference in volume between males and females. Further, Chancellor et al. (2011) found birds at more extreme latitudes have more hippocampal neurogenesis (measured by DCX-ir) than birds in more moderate environments; they posit that these data affirm that birds in harsher climates and environments have increased hippocampal neurogenesis to support an increased reliance on food-storing and retrieval in harsher environments; this speculation is sometimes referred to as the "environmental harshness hypothesis", not dissimilar from the "climate harshness hypothesis" (Currie et al., 2004). But, as in Roth et al. (2011), Chancellor et al. (2011) pooled data from males and females prior to analyzing data for study site differences. Unfortunately, neither study report whether an interaction between sex and study site were detected; the reasons for this are unclear, as the results of such an interaction would greatly clarify (by either corroborating or refuting) the purported data described by Lee et al. (1998) showing a female-biased sex difference in spatial memory in Alaskan, but not Coloradan chickadees.

There are parallels between how spatial behaviour and the hippocampus change between different populations of chickadees along a latitudinal or environmental gradient, and how spatial behaviour and the hippocampus change seasonally: the former varies with place, the other varies with time of year. Indeed, some consider evidence from both types of study (i.e., latitudinal/environmental differences and seasonal studies) not as separate, unrelated factors, but rather complementary lines of evidence to show how overall environmental change (broadly defined in the context of "environmental harshness"; LaDage, 2015) affects the brain and behaviour of food-storing birds. Birds in harsh conditions (be it winter in the context of seasonal studies, or at more extreme latitudes, in the context of latitudinal studies) rely more on hippocampus-dependent spatial memory for survival, and by extension have more specialized hippocampi (i.e., larger overall volume and/or increased neurogenesis) to support this increased demand on memory. In less harsh conditions (be it in spring/summer in context of seasonal studies; or in more moderate/less extreme climates), the demand on spatial memory to retrieve food caches is less so than in winter, and as a consequence, the neural structures supporting memory adapt to accommodate the lesser demand.

The data reported here similarly show that in harsher environments (i.e., winter), females have increased hippocampal neurogenesis compared to females in less harsh (i.e., spring, summer) environments (Figure 15). While I did not examine any behavioural measurements (i.e., performance on a spatial task, as in Pravosudov and Clayton, 2002); the findings observed in this study may be because of an increased reliance on hippocampus-dependent spatial memory as a result of one or a combination of social (i.e., dominance hierarchies), physiological (i.e., body condition and energetics), and environmental (i.e., harshness) pressures borne only by females in the winter, as speculated by Petersen and Sherry (1996). Conversely, this sex difference may not be detectable (if existent at all) in more favorable, less-harsh environments (i.e., spring). Indeed, this is exactly the mechanistic context in which Lee et al. (1998) describe their data showing sex differences in spatial memory in Alaskan, but not Coloradan birds.

Given the lack of clarity in prior studies as to how and whether sex may interact with the effect of environmental harshness on brain and behaviour in food-storing birds, much more study is needed. One such approach might be to examine both seasonal

behavioural change and neuroanatomical change in the same population of wild birds, taking care to specifically examine sex differences in both behavioural and neural measures. In the context of food-storing birds, such an approach has only been accomplished by Lange et al. (2021) in a relatively small sample of Eurasian willow tits and great tits. While such an approach, particularly as it relates to behavioural observations of sexually monomorphic species (such as chickadees) in the field may be difficult, recent advances in radio frequency identification tags (RFID; e.g., Bridge et al., 2019) and the use of these tags in sophisticated behavioural arrays located in the natural environment (including in recent studies of food-storing birds, e.g., Branch et al., 2022; Sonnenberg et al., 2022) may mitigate some of these potential difficulties and prove beneficial in the study of sex differences in brain and behaviour using a larger sample of wild birds without the confound of captivity.

3.3 THE ROLE OF CAPTIVITY IN BEHAVIOURAL AND NEUROBIOLOGICAL STUDIES OF WILD BIRDS

In their seasonal study of Eurasian willow tits and great tits, Lange et al. (2021) argued that any studies aiming to show evidence of seasonal neural plasticity in wild birds cannot achieve those aims if their subjects are kept in captivity for any portion of the experiment (see Pravosudov, 2022 for rebuttal). As such, in their study, Lange et al. (2021) sacrificed their birds in the field, shortly after capture. Similarly, this study was designed to minimize the potentially confounding effects of captivity on the hippocampus of food-storing birds (reviewed in Phillmore et al., 2022). Here, birds were not housed in any form of laboratory-based captivity and were sacrificed shortly (within ca. 1 hour) after capture. Further, I did not quantify neurogenesis using an exogenous label (e.g., BrdU) that would necessitate some period of captivity to allow the label to become incorporated into dividing cells (e.g., Hoshooley et al. 2007); I opted instead to quantify neurogenesis using DCX, an endogenous label which can be used to examine immature neurons in post-mortem tissue.

It is unclear what role captivity (or lack thereof) played in this study, as the data is neither entirely consistent nor entirely inconsistent with prior studies of either captive or non-captive chickadees. For example, I was able to replicate the findings of Smulders

(2002; using data from Smulders et al., 1995; a non-captive study) as it related to brain shrinkage during breeding season. I also replicated the findings of Phillmore et al. (2006; using data from the same birds in Hoshooley et al., 2007; a captive study) as it related to brain shrinkage in late summer-fall. However, my data also diverge from these studies, in that some evidence of shrinkage was also observed in the brains of birds captured in December (Figure 9).

Further, the data presented here showing changes in neurogenesis with a peak (in females) in January (Figure 15), were in line with the trends observed by Hoshooley et al. (2007) in captive birds. However, Hoshooley et al (2007) only used male subjects in their study; therefore, in the context of the sex differences observed in this study (female-bias), these findings were actually inconsistent with the data from Hoshooley et al. (2007). Moreover, the lack of findings as it relates to change in hippocampal volume was inconsistent not only with prior non-captive studies (e.g., Smulders et al., 1995; Lange et al., 2021), but also with prior captive studies (e.g., Hoshooley and Sherry, 2007).

Overall, the mixed evidence shown in this study may lead some to suggest that captivity may not actually play a major role in affecting seasonal regulation of hippocampal plasticity in food-storing birds. However, as previously described here and in other sources (e.g., Phillmore et al., 2022), using captive birds in studies of brain and behaviour severely limits the conclusions one can draw, particularly in the context of ecologically relevant behaviours and their neurobiological correlates in wild birds.

3.4 LIMITATIONS

A primary limitation of this study is associated with the dates I used to capture birds: I anticipated capturing birds across the year in three groups, according to the three photoperiodic conditions: photostimulated, photorefractory, photosensitive. However, gonad data revealed very few of the predicted “photostimulated” birds showed the gonadal indicators of photostimulation; indeed only 3/16 birds captured in this group showed any evidence of photostimulation, and likely these birds were only partially photostimulated (Figure 7).

Here, I used only gonad size or stage to identify photoperiodic condition in the subjects in this study. Prior research has additionally quantified some of the “upstream”

endocrine regulators of gonadal recrudescence as a complementary indicator of photostimulation. For example, Phillmore et al. (2005) used radioimmunoassay to quantify changes in plasma LH collected from captive black-capped chickadees at various time points over a ca. 80-day experiment where photoperiod was manipulated to examine absolute photorefractoriness. While I did not quantify any upstream endocrine regulators of gonadal development (e.g., FSH, LH) here, such an approach would, in the context of this project, provide clarification as to the degree of photostimulation in the spring group. Given that the dates in which I captured the predicted photostimulated birds occurred after the critical day length threshold for initial photostimulation, it may be that these birds had elevated levels of LH, but simply did not yet show the gonadal indicators of photostimulation. In a larger context, such an approach would also shed more light on the fine-scale neurobiological changes that occur in response to changes in photoperiod, not only in the hippocampus, but also in other regions (e.g., vocal control system). For example, this would allow us to potentially examine whether the downregulation of hippocampal neurogenesis observed between winter and spring (shown here in female chickadees; Figure 17) occurs independently of the upregulation of LH (and associated gonadal recrudescence). Similarly, but perhaps more interestingly, using such measures would allow us to examine whether the rapid growth in vocal control nuclei observed across many songbird species in spring, occur entirely post-gonadal maturation, or in tandem with gonadal maturation. While it is generally believed that the behavioural, physiological and neurobiological changes associated with photostimulation in birds occur in a "bottom-up" manner (i.e., gonad development occurs first, and it is the subsequent spike in gonadal steroids drive the rapid of the vocal control system in spring; reviewed in Tramontin and Brenowitz, 2000; Rose et al., 2022), the evidence for this is mostly speculative and unclear.

Another limitation associated with this study is the variation in sample size across photoperiodic conditions/seasons; while variation in sample size in studies of wild birds is generally not uncommon, Pravosudov (2022) suggests that using small sample sizes (defined in this commentary as < 24 birds per group) when quantifying seasonal change in brain and behaviour in food-storing birds exacerbates the already-present sampling biases due to the non-random approaches used to capture birds. For example, usually in

neurobiological studies of wild birds, the *N* birds which comprise a particular study sample are, for ethical and practical considerations, most often simply the first *N* birds which are captured. But, as Pravosudov (2022) suggests, the methods used to attract wild birds to traps (e.g., song playback, decoys) may attract a select group of birds which show unrepresentative behavioural or physical characteristics relative to the broader population – for example, more aggressive chickadees may respond quicker to playback or to a decoy, or leaner chickadees (i.e., poorer body condition) may respond more quickly to a trap baited with seeds – which could lead to these behavioural characteristics being overrepresented in the study sample, potentially affecting the data and conclusions derived from a particular study (Pravosudov, 2022).

Here, the variability in sample sizes can largely be attributed to the exclusion of juvenile birds from analyses: because it is well-documented that the same brain attributes examined in this study for seasonal variation also vary with age in songbirds (e.g., Smulders et al., 1995; 2000), I was forced to exclude data from all juvenile birds to avoid introducing the confound of age into my design. I also could not reasonably include age as a covariate in my design (e.g., Smulders et al., 1995), as there were no juveniles in the spring group and only one juvenile in the winter group.

So, how did I capture so many juveniles? I relied on the age-distinguishing field marks described by Pyle et al. (1997) to age birds in the field, considering a combination of factors such as shape, wear, and color of the outer rectrices and overall texture of body feathers. I also considered the color of the upper mandible (i.e., roof of the mouth) lining, given that Pyle et al. (1997) suggest that the color of the lining in black-capped chickadees varies with age, as in the closely related mountain chickadee and tufted titmouse (*Baeolophus bicolor*). Pyle et al. (1997) state that "most [juvenile] birds may be separated from [adults] through fall by the dark gray roof of the mouth […], *vs* white in [adults]" (p. 338). However, using museum specimen data, Renaud et al. (2007) dispute the age-related field marks in Pyle, finding that mandible lining was a weak indicator of age in black-capped chickadees, as both juvenile and adult birds showed evidence of dark and light linings. Fylling et al. (2018) used bird banding data (including complementary skull pneumatization data) to further refute (and ultimately disprove entirely) the descriptions of Pyle et al. (1997); they showed that black-capped chickadees actually

display the opposite age-associated mandible lining patterns than mountain chickadees: instead of a dark lining in juveniles and a light lining in adults (as in mountain chickadees), juvenile black-capped chickadees show a light lining, while adults show a dark lining.

I therefore assume that the main reason behind why so many juvenile birds were captured during fieldwork was that I relied on a (now largely disproven) field indicator of age when aging subjects for this study. But even so, this still does not fully resolve the issue, given that I used the same field marks (including mandible lining) to age birds in the field in the subsequent winter group, with markedly greater success: the winter group consisted of only one juvenile (versus 11 juveniles in the summer group).

Ultimately, future studies should be mindful of using mandible lining (at minimum, the descriptors of mandible lining described by Pyle et al., 1997) as a reliable indicator of age in black-capped chickadees. Prior seasonal studies in chickadees (e.g., Barnea and Nottebohm, 1994; Hoshooley et al., 2007) have controlled for age in their sample by capturing individuals from a banded population for which age can be verified or inferred based on banding data (i.e., any birds banded over a year prior to capture will be after-hatch year birds according to the calendar-based system, regardless of their age at the time of capture); such an approach might prove beneficial in future studies on this topic.

3.5 IMPLICATIONS AND CONCLUDING REMARKS

In summary, while these data, particularly as it relates to female-specific seasonal variation in hippocampal neurogenesis, may have significant implications in how females are studied in the context of future studies of brain and behaviour in food-storing birds, replication of these findings in an equally large or larger sample is required. Complimentary evidence from behavioural studies would prove useful in confirming these findings. Overall, the results of this study, when taken together, underscore not only the value, but also the complexity, of taking multiple approaches to quantifying neural plasticity, and ultimately, probing the interaction between brain and behaviour in wild vertebrates.

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