Neurobiological and Epigenetic Substrates of Learning, Memory, and Behavioural Flexibility in Three Songbird Species: the Black-capped Chickadee (*Poecile atricapillus*), Zebra Finch (*Taeniopygia castanotis*), and European Starling (*Sturnus vulgaris*)

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In loving memory of Olivia Quinn Bibby

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ABSTRACT

Songbirds rely on learning, perceiving, and producing complex vocalizations to perform critical functions such as conspecific identification, mate selection, and territory defence These cognitive processes are influenced by a variety of natural and laboratory-induced factors, including sex, season, and social context. Along with changes in behaviour, songbirds have been extensively studied for the neurobiological mechanisms underlying their vocal behaviour, making them an ideal model to investigate both behavioural and neural plasticity. This thesis aims to explore the intricate relationship between neurogenesis, learning, and perception in songbirds, specifically examining the impact of acoustic discrimination, social isolation, and season on neurogenic and epigenetic processes. Employing a comparative cognitive approach, I generated and tested hypotheses regarding cognitive and behavioural adaptations in three songbird species: black-capped chickadees, zebra finches, and European starlings. In the first study, I examined DNA methylation levels in key vocal and auditory brain regions of all three species, thereby providing insights into the potential role of epigenetic processes in behavioural and neural plasticity in songbirds. In the second study, I examined the effects of social isolation and auditory discrimination on neurogenesis in auditory brain regions. In the third study, I explored seasonal changes in the rates of neurogenesis in European starlings, specifically regarding vocal behaviour, conspecific discrimination, and stress. Overall, results from the studies described in this thesis lend new insight into how epigenetic factors may mediate behavioural and neural plasticity while extending the literature examining how learning, perception, and an animal's environment modulate neuroplasticity in specific brain regions.

LIST OF ABBREVIATIONS AND SYMBOLS USED

Units

%Cov	percent coverage
°C	degrees Celsius
cm	centimetre
dB	decibel
h	hour
Khz	kilohertz
min	minute
ml	milliliter
ml	millilitre
mm	millimetre
ms	millisecond
η^2p	partial eta squared
p	p-value
pg	picogram
s	second

Chemicals, Molecules, and Sundry

5-mC	5-methylcytosine
AFB	anterior forebrain pathway
AHN	adult hippocampal neurogenesis
ANOVA	analysis of variance
ASY	after second year
BrdU	bromodeoxyuridine
CMM	caudomedial mesopallium
CNS	central nervous system
CORT	corticosterone
CWS	Canadian Wildlife Service
DCX	doublecortin
DG	dentate gyrus

DNMT	DNA methyltransferases
DR	discrimination ratio
ELISA	enzyme-linked immunosorbent assay
f	fusiform
fDR	final discrimination ratio
FOV	field of view
GnRH	gonadotropin-releasing hormone
GO	rewarded stimulus
HA	hyperpallium apicale
hDR	highest discrimination ratio
HLR	heterophil:lymphocyte ratio
Hp	hippocampus
HPd	dorsal region of the hippocampus
HPv	ventral region of the hippocampus
HRM	Halifax Regional Municipality
IEG	immediate early gene
IHC	immunohistochemistry
ir	immunoreactive
LMAN	lateral magnocellular nucleus of the anterior nidopallium
LPS	lamino pallio-subpallialis
MOM	mouse-on-mouse
Mp	multipolar
NC	nidopallium caudale
NCM	caudal nidopallium
NCMd	dorsal region of the caudal nidopallium
NCMv	ventral region of the caudal nidopallium
ng	nanogram
NoGO	unrewarded stimulus
PBS	phosphate buffered saline
PBS/T	phosphate buffered saline with Triton

PDP	posterior descending pathway
r	round
RA	robust nucleus of the archistriatum
SGZ	subgranular zone
SVZ	subventricular zone
SY	second year
TET1	ten-eleven translocation methylcytosine dioxygenase 1
TNL	tone no light
TPL	tone plus light
UCLA	University Committee on Laboratory Animals
VZ	ventricular zone
WBC	white blood cell

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CHAPTER 1: GENERAL INTRODUCTION

Songbirds have captured the interest of researchers from across the academic disciplines for more than a century (Baker, 2001). Building on the work of naturalists dating as far back as the 1700s, by the mid-20th century, a more formal understanding of songbird behaviour developed through deliberate observation. What began as a fascination with birdsong turned into a burgeoning research field whose findings have contributed to the wider scientific community's understanding of phenomena like language development, auditory perception, and spatial memory (Nottebohm et al., 1990; Healy et al, 2005). Alongside the expanding behavioural literature, research has uncovered the neural mechanisms by which the diverse, specialized behaviours of various songbird species are realized (Willbrecht and Creanza, 2003). In this thesis, I examined some of the neurobiological correlates of adult auditory learning and perception in three songbird species: zebra finches (*Taeniopygia castanotis*), European starlings (*Sturnus vulgaris*), and black-capped chickadees (*Poecile atricapillus*).

1.1 Species Diversity in Birdsong Learning

Young songbirds learn song from a tutor, typically their father, during early development—a process similar to that of human language acquisition (Nottebohm et al., 1990). The sensitive period, an optimal time during development for vocal learning, is a well-established phenomenon and when leveraged in scientific research has brought insight into how social, environmental, and genetic factors affect language learning and production (Beecher, 2017; Searcy & Nowicki, 2019; Woodgate et al., 2011; Scharff & Adam, 2013). Many songbirds are restricted to song learning during early development,

but others are open-ended learners, meaning they continue learning as adults by adding new songs to their repertoires annually, or by modifying existing ones (Chaiken & Bohner, 2007). Open-ended learning is thought to be a product of sexual selection, where females prefer males with larger vocal repertoires, and thus, males with larger repertoires have greater reproductive success than males with smaller ones (Catchpole & Slater 2008). In the case of male intrasexual competition, however, a simpler song confers an adaptive advantage (Beecher & Brenowitz 2005). Indeed, males who are defending their territory prefer the familiar songs of neighbouring males, as opposed to newcomers who have yet to establish their territories. This preference for neighbouring males allows birds to focus on breeding behaviours like foraging without incurring costs associated with frequent aggressive encounters (Beecher & Brenowitz, 2005).

The propensity for song to signal information about mate quality and for recognition means that members of the same species (conspecifics) must be able to receive and interpret those signals. Song perception, too, has been studied thoroughly in songbirds, investigating what features of birdsong are perceived by conspecifics, how well those features can be discriminated, and what adaptive benefit superior perception confers (Figure 1.1). The diversity of specialized behaviours in songbirds offers researchers a wealth of opportunity to learn from several research paradigms, and to use a comparative approach. In this thesis, I chose to study several species, each with different strengths and weaknesses, but ultimately providing complementary perspectives on the topics at hand. Together, the studies encompass a comparative cognitive approach, where hypotheses are generated and tested with respect to each species' cognitive and behavioural adaptations.

1.1.2 Zebra Finch

Zebra finches are an ideal model for studying vocal learning and production (Mello, 2014) as they are tractable in the laboratory setting, breed relatively easily, and mature to adulthood in 90 days (i.e., 3-months), a relatively short time. In songbirds like the zebra finch, the male's song consists of sequences of song elements or notes called syllables and range in length from milliseconds to several seconds, but females do not sing a learned song (Glaze & Troyer, 2004). Male zebra finches learn songs from their father, or "tutor", beginning during a sensitive phase in early development and is characterized by three distinct but overlapping periods: the sensory, the sensorimotor, and the crystallization periods (Slater, Eals, & Clayton, 1988). During the sensory period, birds listen to and memorize song from their tutor. After about 30 days (i.e., 1-month) post-hatch, the sensorimotor phase begins, where birds start to vocalize more robustly (i.e., beyond begging calls) and produce "subsong"—a plastic song that approximates the tutor's song (Roper and Zann, 2006). The crystallization period starts around 60 days (i.e., 2-months) post-hatch, with subsong becoming less variable, and by 90 days (i.e., 3-months) post-hatch, the bird will have honed production of each syllable and order of syllables into its stable, or "crystalized" song (Adret et al., 2012, Chen et al., 2017, Braaten, 2010; see Figure 1.3 for a spectrogram of typical zebra finch song). The learned song is highly stereotyped, and adults do not significantly modify their songs after the crystallization period—a hallmark of closed-ended learners. This learning process is analogous to the way human infants learn language; both birdsong and speech require listening to an adult model, practicing the vocal behaviour, and eventually, crystallization (See Phillmore & Tsang, 2020 for review). Unsurprisingly, both humans and birds have

dedicated neural regions for auditory perception and memory, motor production, and sensorimotor integration, thereby making the zebra finch an ideal model for studying aspects of language learning and production, their neurobiological substrates, and the environmental factors that modulate them (Tchernichovski & Marcus, 2014; Mooney, 2009; Leitner, 2007, see more below).

Along with song learning, researchers have also investigated song perception (Theunissen & Shaevitz, 2007). In zebra finches, song complexity—the number of song elements within a song phrase—seems to confer a competitive mating advantage; indeed, males who can accurately learn accurate copies of complex songs from their tutors are more likely to reproduce than males who do not (Williams, Kilander, & Sotanski, 1993). Although female finches do not sing, they prefer males who have more complex songs, and to identify high quality mates, they must be able to accurately discriminate between males' songs (Honarmand, Riebel, & Naguib, 2015; Sewall et al., 2018). Song complexity, however, is not the only factor that determines the perceived quality of male birds – physiological features such as plumage ornaments, such as cheek patches or bill colour are also attended to by females; there is still much to be learned about the basis and application of female perception of male song (Hauber, Campbell, & Woolley, 2010). Indeed, the function of female perception of male song outside of sexual attraction remains unclear, although studies show that female zebra finches show preferences for song produced by males unfit for reproduction, if the song is similar to their fathers (Riebel, 2000). Further, social feedback provided by females during males' song development also appears to facilitate their song learning (Caruso-Peck & Goldstein, 2019) (Figure 1.1).

1.1.3 European Starling

Like zebra finches, European starlings learn their song from a tutor in early life; however, unlike zebra finches, who are close-ended learners, starlings are open-ended learners who will continually adjust their vocal repertoire over their lifetime: adding some phrases and dropping others annually (Eens, 1997; see Figure 1.4 for a spectrogram of typical European starling song). Although primarily a male behaviour in starlings, female starlings (unlike zebra finches) also learn and produce birdsong, but their songs are generally shorter and less complex than their male counterparts (Pavlova, Pinxten, & Eens, 2005). In starlings, birdsong functions in mate-attraction, territoriality, flock cohesion, and maintenance of dominance hierarchies, but the quality of the song and rate of singing differs depending on context (Eens, 1997). For example, male birds sing less complex song when defending their nest boxes from other males than when attempting to attract females, and males' song bout frequency decreases once they have successfully attracted a mate (Mountjoy & Lemon, 1991; Eens, Pinxton, & Verheyen, 1993). Females also use their song for territoriality, singing to ward off intruding females, but not males, thereby maintaining the monogamy of their male partner (Pavlova, Pinxten, & Eens, 2007). This does not explain, however, why females continue to sing after the breeding season, though this could function to form and maintain same-sex pairs observed in female starlings. Like finches, female starlings prefer males with longer song bout lengths and larger repertoires (Verheyen, Eens, & Pixton, 1991).

1.1.4 Black-capped Chickadee

Like the above-mentioned species, chickadees require exposure to species-typical song during development to produce species-typical song. Compared to those of the

zebra finch and European starling, black-capped chickadee song is less complex, consisting of a simple, two-note sequence known as the "fee-bee" (Ficken, Ficken, & Witkin, 1978; see Figure 1.5 for a spectrogram of typical black-capped chickadee "fee-bee"), however they are able to introduce a level of variability by pitch-shifting the absolute frequency of the notes while maintaining relative frequency (Weisman et al., 1990). Shifting occurs depending on social context (Hill & Lein, 1987): male chickadees singing a lower-frequency fee-bee during territorial behaviour than when attracting mates (Hill & Lein, 1987). Territorial males will also pitch shift to match the song of intruders, which is thought to indicate willingness to escalate an aggressive encounter (Otter et al., 2002). Once an aggressive encounter is escalated, this lower-frequency song may be abandoned in favour of other complex vocalizations such as gargles – a short call typically associated with aggressive encounters (Baker, Wilson, & Mennill, 2012).

Like European starlings, male black-capped chickadees show a relative increase in singing behaviour when courting mates than when paired with one (Otter & Ratcliffe, 1993) and chickadees use their song for territoriality (Baker, Wilson, & Mennill, 2012). These behavioural changes occur within a seasonal context: singing increases in spring when males court mates and defend their territories, and decreases in the fall and winter, when the breeding season is over (Phillmore et al., 2006; Avey, Rodriguez, & Sturdy, 2011). Singing in female chickadees has been observed widely, and research has uncovered an acoustic discrepancy between male and female song, which is likely the basis by which birds discriminate between males and females using vocalizations (Hahn, Krysler, & Sturdy, 2013; Montenegro et al., 2020).

Unlike zebra finches and European starlings, black-capped chickadees are a food-storing species, storing food in many thousands of caches in autumn and retrieving them throughout the winter when food sources are otherwise scarce (Sherry, 1984). In addition to their value as a model of vocal communication, chickadees have also been widely studied as a spatial memory expert (Pravosudov & Clayton, 2002). These seasonal shifts in behaviour, fluctuating between breeding behaviours including singing and spatial, food storing behaviours, make black-capped chickadees a particularly interesting model to study behavioural and neural plasticity. Indeed, many studies have investigated changes to the neural substrates of these behaviours across season in laboratory and field studies (e.g., Sherry, 1984; MacDougall-Shackleton et al., 2005; Phillmore et al., 2006; Hoshooley et al., 2007; Shettleworth, Hampton, & Westwood, 1995; see Table 1.1 for a comparison table of the study species).

1.2 Neurobiology of Birdsong

Given the remarkable behavioural plasticity exhibited by many songbird species, extensive research has sought to understand the neurobiological mechanisms that govern and modulate these behavioural specializations. The following sections provide an overview of the neurobiological correlates underpinning song learning, production, and perception.

1.2.1 The Vocal Control System

Vocal learning and production in songbirds are under control of a discrete series of nuclei referred to as the vocal control system (Nottebohm, 2005) (Figure 1.2). The vocal control system consists of two major pathways: The anterior forebrain pathway

(AFP) and the posterior descending pathway (PDP). Although active during vocal production, the AFB primarily supports song learning, while the PDP is responsible for both song learning and production. Both pathways originate in HVC (not an abbreviation): In the PDP, HVC projects directly to the robust nucleus of the archistriatum (RA), whereas in the AFP the HVC connects indirectly to RA via Area X, DLM, and LMAN, to allow modification of song during vocal learning. Research has revealed structural and functional similarities between song system nuclei and their pathways and those of mammalian motor circuits involving the cortex, thalamus, and basal ganglia (Nottebohm, Stokes, & Leonard, 1976; Paton, Manogue, & Nottebohm, 1981; Vates, Vicario, & Nottebohm, 1997).

In addition to HVC, two discrete forebrain nuclei receive auditory information via a third, ascending auditory pathway and are involved in the perception and discrimination of hetero- and conspecific song: the caudomedial nidopallium (NCM) and the caudomedial mesopallium (CMM) (Mello & Clayton, 1994). Like HVC, NCM has been implicated in forming memory of an adult tutor (i.e., a song "template") during song learning; however, both NCM and CMM also appear to be important for the formation of long-term memories of conspecific songs in adulthood. Evidence for the function of these regions comes from studies using labeling of immediate-early gene (IEG) expression, specifically early growth response 1 (EGR1; also known as NGFI-A, TIS8, Krox24, *zif*/268, and *ZENK*) and using electrophysiology. There is a rapid upregulation of ZENK mRNA expression and lagging ZENK protein levels in these regions following adult birds' exposure to playback of birdsong, suggesting that song, but not irrelevant acoustic stimuli, is sufficient to induce rapid changes in gene expression in these regions—a

finding corroborated by electrophysiological studies (Chew et al., 1995; Mello & Riberiro, 1998). Further, observed responses in these regions habituate quickly, suggesting birds' ability to discriminate novel song stimuli. Upon presentation of a novel song stimulus, an increase in ZENK mRNA is observed within seconds, with peak levels reached within 30-45 min (Mello, Nottebohm, & Clayton, 1995). Overall, the temporal dynamics of ZENK protein levels in NCM and CMM following birdsong exposure provide valuable insights into the rapid molecular changes associated with auditory perception and memory formation in these regions.

1.3 Neuroplasticity

Neuroplasticity is a phenomenon whereby neural tissue of the central nervous system (CNS) undergoes growth and reorganization (Fuchs & Flügge, 2014). Research on neuroplasticity has expanded immensely over the past two decades, with much research finding its way into the realm of popular science (Doige, 2007; Falk et al., 2013; Douyon, 2019). Findings from research in this field are compelling because they offer insight about the brain's capacity for change, thereby overturning previously held beliefs about the limits of adult learning and memory. Not surprisingly, neuroplasticity has become a mainstay topic across academic disciplines, including psychology, neuroscience, health science, education, and medicine (Brookman-Byrne & Thomas, 2018; Dinse, 2020; Frankenhuis & Nettle, 2020). To date, findings from research on neuroplasticity have been fruitful, offering insight to fundamental medical questions about brain and spinal injury and neurodegenerative diseases. In the behavioural sciences, neuroplasticity is studied for its role in allowing animals to adapt to changing environments that require reliance on different cognitive abilities, which requires changes in gene expression. The

potential applications though are much broader, with neuroplasticity being considered in such realms as sport and education (Abraham, 2022).

1.3.1 Neuroplasticity in the CNS

Growth and reorganization in the CNS arise through several processes, including changes to the number, size, structure, and function of neurons (Carcini 1999). One such process is neurogenesis, which involves the birth of neuronal precursor cells in the CNS and their subsequent differentiation, recruitment, and integration to neural networks, which when activated, perform a specific behavioural or cognitive function (Gage, 2002; Götz & Huttner, 2005). Neurogenesis has primarily been considered a developmental process; that is, rates of neurogenesis are at their highest in developing animals and are subsequently culled through a form of cell death called apoptosis. According to this perspective, neurons are initially overpopulated in CNS, and as neural networks become specified, cells not required for efficient communication are eliminated. This view of neurogenesis, however, is somewhat bleak; it implies that there are limits to which the brain can growth or change in later life. Although there are limits, seminal findings about neurogenesis that emerged in the 60s and caught wind in the 80s inspired a new wave of thinking about neurogenesis in adult animals (Owji & Shoja, 2020), including in adult songbirds. Neuroplasticity in the CNS is now widely recognized as a dynamic and ongoing process that occurs throughout life, demonstrating the brain's remarkable ability to adapt to various environmental and experiential factors.

1.3.2 Early Research on Adult Neurogenesis

Early evidence of neurogenesis in the adult mammalian brain was forwarded in a series of studies by Josef Altman (see Altman, 1962 – 1969 and Altman & Das, 1965). In these studies, the authors used radiolabeled thymidine ([3H]thymidine) to assess cell proliferation in the brains of rats up to several months old (1962), and later, in adult rats and cats (1963). This autoradiographic technique would later be combined with histological stains to further demonstrate the presence of proliferating and migrating adult-born cells. By 1969, Altman and Das had established a theory that the subependymal zone of the lateral ventricle was a site for proliferating neurons, which then traveled along the "rostral migratory stream" to replace cells in the olfactory bulbs (1969). Despite the success of these studies, due to the sociopolitical culture surrounding the research at the time, findings were largely ignored, and the topic of adult neurogenesis remained quiet until revisited in the 1980s (see Owji and Shoja, 2020 for a review).

1.3.3 Adult Neurogenesis in Birds

The first report of adult neurogenesis in birds came from Goldman and Nottebohm (1983), who used [3H]thymidine–a radioactive version of the DNA base, thymidine–to detect adult born neurons in the ventricular zone (VZ) of HVC. In subsequent studies, the authors found that neurons were born in the VZ and migrated to HVC via radial glia where they would form functional connections to robust nucleus of the arcopallium (RA) or become interneurons (Nottebohm & Alvarez-Buylla, 1993). Eventually, a picture began to form of the processes by which new neurons proliferate in VZ "hotspots", migrate along radial glia, and are recruited to different brain regions (Barnea &

Nottebohm, 1994; Kirn & Nottebohm, 1993). Evidence for neuronal turnover in target regions, specifically, HVC, developed, explaining how new neurons replaced old ones, thereby maintaining total neuron number within target regions Alvarez-Buylla et al., 1992; Kirn & Nottebohm, 1993).

Adult neurogenesis in birds is not restricted to HVC; in fact, adult born neurons migrate and differentiate into mature neurons when they reach their target destinations throughout the forebrain (Alvarez-Buylla & Nottebohm, 1988). This contrasts with findings from adult neurogenesis in mammals, which is limited to several neurogenic niches; namely, the dentate gyrus of the hippocampus (DG), the subventricular zone of the lateral ventricle (SVZ), and the olfactory bulbs (Ming & Song, 2011). Like mammals, however, adult neurogenesis in birds is associated with learning and memory (Deng, Aimone, & Gage, 2010; Barnea & Pravosudov, 2011). Indeed, research has revealed relationships between adult neurogenesis and performance of specialized behaviours, including vocal learning and spatial memory. For example, in zebra finches, neuronal recruitment to nidopallium caudale (NC) is associated with adults memorizing the vocalizations of their offspring before they fledge, and in black capped chickadees, hippocampal neurogenesis is necessary for successful caching and retrieval behaviour—a hippocampus-dependent, spatial memory task (Barkan et al., 2007; Hall, Delaney, & Sherry, 2014). The ease with which neurogenesis can be observed and measured in birds makes them an ideal model for studying the neurobiological substrates of learning and memory.

Since performance of specialized behaviours like vocal learning and production and caching and retrieval varies across season, it is perhaps unsurprising that rates of

neurogenesis, which appear to underlie these behaviours, is also subject to environmental factors like season. For example, in male brow-headed cowbirds (*Molothrus ater*) and red-winged blackbirds (*Agelaius phoeniceus*), rates of neurogenesis are higher in post-breeding condition than in breeding condition, which coincides with when their songs are most plastic (Guigueno, Sherry, & MacDougall-Shickleton, 2016). And in black-capped chickadees, hippocampal neurogenesis peaks in October, which coincides with when birds are forming spatial memories of cache locations (Barnea & Nottebohm, 1994). By manipulating photoperiod and hormones, lab studies have also revealed "seasonal" changes in measures of neurogenesis; however, findings from lab and field studies do not always align (Calisi & Bentley, 2009). The propensity of some aspects of birds' physical environments over others remains elusive, and the effect of captivity on baseline rates of neurogenesis may obscure results (Aitken, 2015; Phillmore et al., 2022).

1.3.4 Labeling Neurogenesis – Exogenous vs. Endogenous Markers

Cells undergoing neurogenesis can be labeled using exogenous and/or endogenous markers. Though exogenous markers like [3H]thymidine offer the benefit of tracking adult born neurons from the time of injection, they introduce logistical problems for experimenters who must inject subjects with the label, usually over a period of several days (Gould & Gross, 2002). Further, for studies of free-flying subjects, there is risk for introducing the toxic substances into the wild. In any case, exogenous markers can damage cellular DNA, so endogenous markers may be preferred where the precise timing of cell birth is not required. Ki-67 and doublecortin (DCX) are two markers of neurogenesis that can be visualized using classic immunohistochemical techniques (Kuhn & Kuhn, 2007; Wojtowicz & Kee, 2006). Ki-67 is a cellular marker detected in

proliferating cells, whereas DCX is expressed in immature and migrating neurons. In addition to the logistic advantages of using endogenous markers, these markers also visualize cell morphologies, enabling the recognition of cell types and quantification of cellular characteristics (Balthazart & Ball, 2014).

1.4 Neurogenesis and Epigenetics

Epigenetic processes serve as a critical interface between genes and the environment, allowing organisms to acquire heritable phenotypic changes (Covic, Karaca, & Lie, 2010). Behavioural scientists are increasingly interested in studying these processes to understand how environmental and cognitive stimuli can induce enduring changes in brain function and behaviour across generations, without altering the genetic code itself (Colvis et al., 2005). Traditionally, it was believed that genetic factors alone governed changes in behaviour and brain function. However, epigenetic modifications introduce an additional layer of regulation that influences gene expression, thereby shaping neural circuits and behavioural responses. Epigenetic mechanisms, such as DNA methylation and histone modification, offer valuable insights into how animals adapt to dynamic environments that require long-term changes in learning and memory (Day & Sweatt, 2011; Molfese, 2011). Furthermore, since epigenetic changes can be reversible, they provide a framework for studying the role of environmental influences in shaping individual differences and adaptive responses. Indeed, neuroepigentic phenomena are salient topics of investigation across the behavioural and life sciences, offering a new layer of insight into the mechanisms and scope of neuroplasticity and associated behaviours (Woldemichael, 2014). Although still a relatively new area of study, recent research has implicated DNA methylation as a mechanism through which gene

expression is altered in the brains of socially isolated songbirds (George et al., 2020). By further untangling the complex interplay among epigenetic processes, neural plasticity, and behavioural responses, neuroepigentic research can shed light on how organisms adapt to dynamic environments, thereby advancing our understanding of the biological underpinnings of behaviour.

1.5 Current Work

My thesis explores the intricate relationship between neurogenesis, learning, and perception in songbirds.

1.5.1 Study 1 – DNA Methylation in the Songbird Brain

The first study focuses on DNA methylation patterns in the songbird brain, comparing the labeling of 5-mC in different brain regions of black-capped chickadees, European starlings, and zebra finches. There are two major research questions associated with this study:

1. Can global DNA methylation patterns be detected using a 5-mC immunohistochemistry protocol adapted from rodents?
2. Are there regional differences in DNA methylation patterns in songbird brains?

The first study is exploratory in nature; however, I expect that the adapted protocol will be sufficient to detect 5-mC in avian brain tissue. Further, given the functional dissociations of brain regions involved in aspects of learning and memory in songbirds, I expect to observe regional differences in global DNA methylation, as is observed in mammalian brain tissue. The objective of this study is to establish a means of detecting 5-

mC in avian brain tissue, which will be a valuable tool in future research on the role of epigenetic processes like DNA methylation in learning and memory. I expect to establish seminal findings about regional differences in DNA methylation, which will serve as a foundation for future research exploring the functional implications of neuroepigentics in learning and memory in songbirds.

1.5.2 Study 2 – Social Isolation, Auditory Discrimination and Neurogenesis

The second study investigates the impact of social isolation on neurogenesis in vocal and auditory brain regions using an auditory discrimination task and analyzing the expression of the marker DCX in HVC, CMM, and NCM. The major research questions addressed are:

1. Does social isolation suppress neurogenesis in auditory perceptual regions in zebra finches?
2. Is mere exposure to auditory stimuli sufficient to prevent suppressing effects of social isolation?
3. Can prolonged engagement in an auditory discrimination task promote neurogenesis in auditory regions?
4. Are brain regions differentially affected by isolation, exposure, and discrimination training?

Given that neurogenesis is a dynamic process influenced by environmental and social factors, I expect the experimental manipulations to have profound effects on the regions of interest. I expect that prolonged engagement in an auditory discrimination task will be sufficient to augment neurogenesis in these regions, because rates of neurogenesis in

these regions are associated with successful task performance. Findings from this study will establish the sensitivity by which neurogenesis can be modulated in auditory perceptual regions, an effect observed readily in other brain regions associated with other aspects of learning and memory, like the hippocampus. Findings from this study will also characterize the dynamics of neurogenesis in CMM, which have not been studied regarding auditory discrimination. This will help generate further research questions and hypotheses about the distinct functional roles of CMM and NCM.

1.5.3 Study 3 – Seasonality and Neurogenesis in the European Starling

Finally, the third study examines the seasonal changes in neurogenesis rates in HVC, CMM, and NCM of European starlings, exploring the relationship between neurogenesis, vocal behaviour, conspecific discrimination, and stress. The research questions addressed in this study are:

1. Are rates of neurogenesis seasonally variable in NCM and CMM of European starlings?
2. Are seasonal changes in rates of neurogenesis associated with seasonal change in stress?
3. Is heterophil to lymphocyte ratio (H:L) associated with seasonal changes in corticosterone, and can this measure be used in place of or to complement corticosterone (CORT) measures?

By comparing neurogenesis across seasons, I will test several hypotheses about the relationships between seasonal behavioural and neural plasticity. By using known behaviour changes as the basis of my predictions, the findings will shed light on what

aspects of behaviour plasticity are supported by neuroplasticity. Findings from this study will build on previous findings from studies using similar design with birds boasting different adaptations and life histories.

Table 1.1 Behavioural characteristics of black-capped chickadee, zebra finch, and European starling

Factors	Species		
	Black-capped chickadee	Zebra finch	European starling
Open-ended learner?	Yes	No	Yes
Food hoarding?	Yes	No	Yes
Sex differences	Males and females are morphologically similar and have similar levels of song complexity.	Males boast elaborate ornamentation and more complex songs than females.	Males boast brighter iridescent plumage compared to females and have more complex songs than them.
Season	Vocal behaviour changes seasonally, with males singing more during breeding season.	Males song more when breeding conditions are favourable.	Vocal behaviour changes seasonally, with males singing more during breeding season.

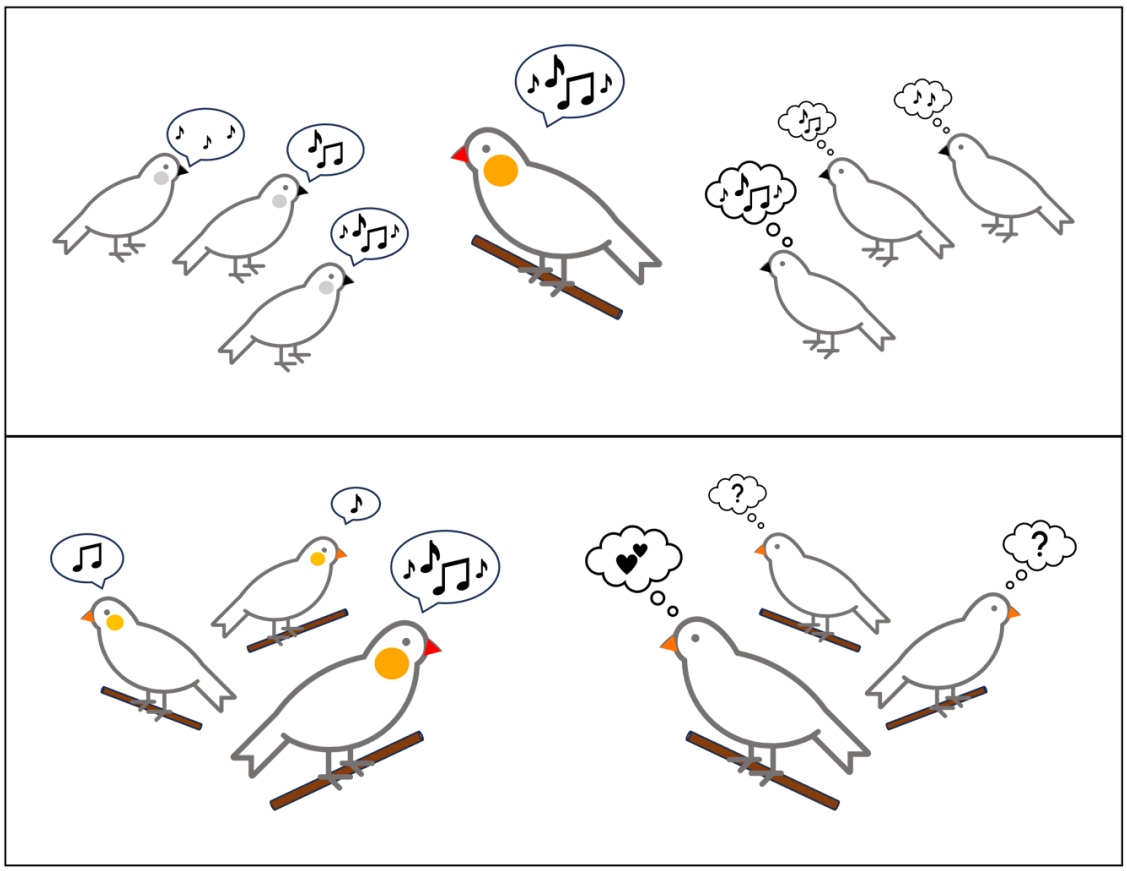

Figure 1.1 Vocal learning and perception in male and female songbirds. Male songbirds who learn complex songs from their tutor (top) – usually their father – have an adaptive advantage over males who learn less complex songs, or males who fail to learn accurate copies of their tutor's song. Females must be able to perceive song complexity to discriminate between conspecifics and develop preferences for high quality mates (bottom).

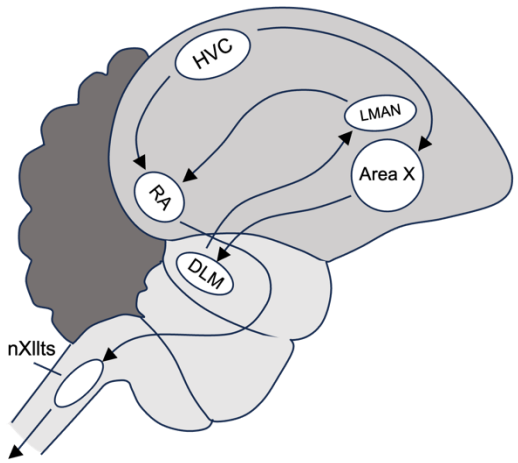

Figure 1.2 Schematic diagram of the vocal control system in songbirds. The vocal control system is responsible for the production and modulation of birdsong. The primary regions include the HVC (used as a proper name), the robust nucleus of the arcopallium (RA), the lateral magnocellular nucleus of the anterior nidopallium (LMAN), Area X, and the dorsolateral nucleus of the medial thalamus (DLM). Adapted from Nottebohm, 2005.

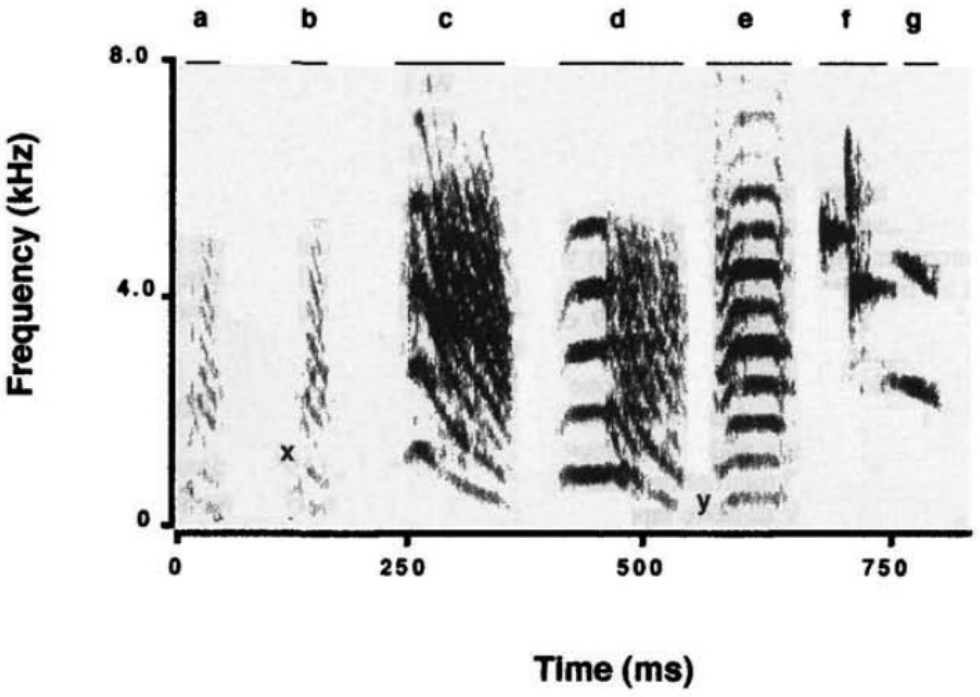

Figure 1.3 Spectrogram of Typical Male Zebra Finch Song. From Sturdy, Phillmore and Weisman, 1999.

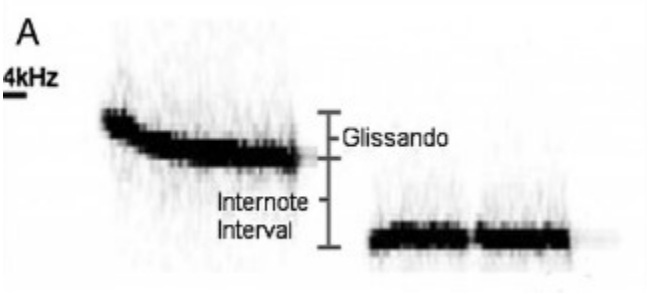

Figure 1.4 Spectrogram of Typical Black-capped Chickadee “fee-bee” Song. From Badcock-Parks and Phillmore, 2022.

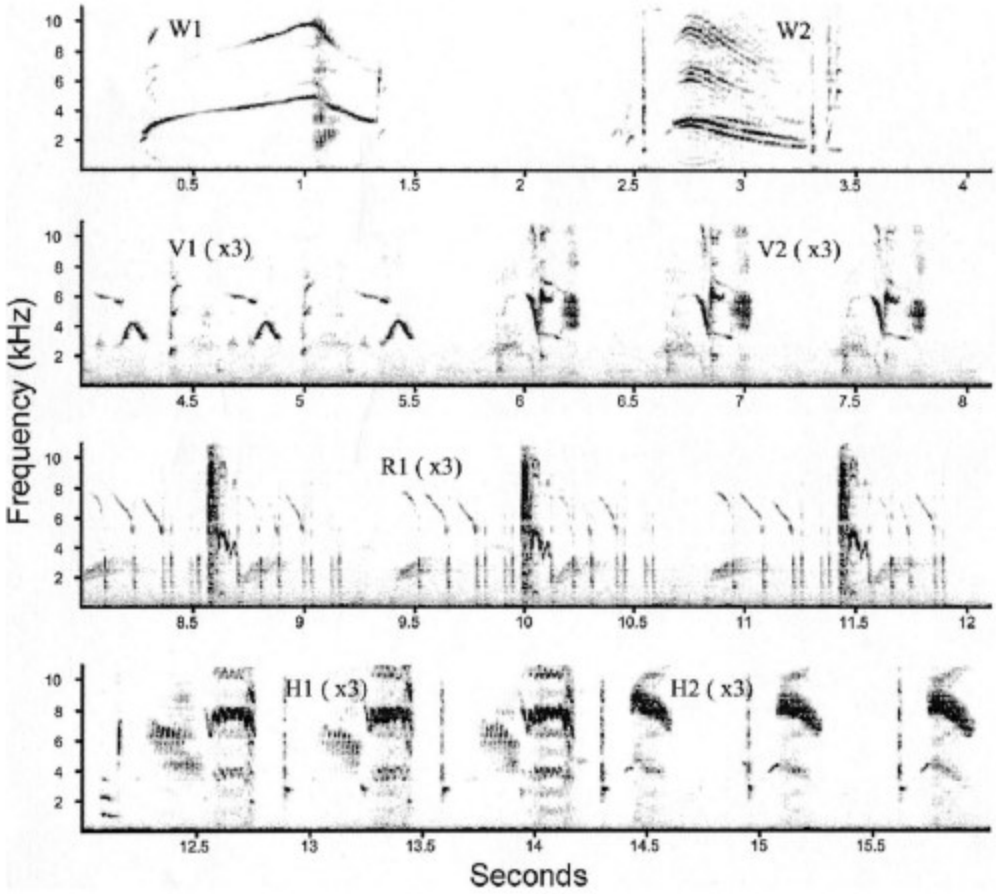

Figure 1.5 Spectrogram of Typical European Starling Song. From Pavlova, Pinxten, & Eens, 2005.

CHAPTER 2: MEASURING GLOBAL DNA METHYLATION IN AUDITORY BRAIN REGIONS OF THREE SONGBIRD SPECIES

2.1 Introduction

Neurogenesis encompasses several processes required to generate functional new neurons, including the proliferation, differentiation, fate specification, and integration of neurons into existing neural circuits (Ming & Song, 2005). These processes are evident throughout development, where neural progenitor cells (NPCs) give rise to neuronal cell types that constitute the central nervous system. NPCs are also present in adult animals, such as birds and mammals, where they continue to proliferate in discrete regions of the brain, like the subgranular zone (SGZ) of the dentate gyrus (DG) in the hippocampus, the subventricular zone (SVZ), and the olfactory bulbs (Kempermann, Song, & Gage, 2015). The maintenance of NPCs and their eventual differentiation into various neural cell types requires regulatory mechanisms that promote and/or silence gene expression to give rise to appropriate cell types: several epigenetic mechanisms have been implicated in the regulation of neurogenic processes (Covic, Karaca, & Lie, 2010).

One of the epigenetic mechanisms that regulates neurogenesis is DNA methylation, which involves the addition of a methyl group to the DNA nucleobase, cytosine, via DNA methyltransferases (DNMTs) (Wang et al., 2015; Figure 2.1. The resulting modified base, 5-methylcytosine (5-mC), represses gene expression by recruiting repressive complexes and blocking the binding of transcription factors and can cause long-term gene silencing—a process critical for determining cells fates in the CNS (Stricker & Götz, 2018). Compared to other tissues, DNA methylation is uniquely patterned in the brain, owing to the requirement for diverse cells fates and gene

expression programs in neurons (Kinde et al., 2015). In attempting to elucidate the role of methylation in adult neurogenesis, much research has focused specifically on the hippocampus, where adult neurogenesis is localized in mammalian models. To date, research has established the roles of several DNA methylation-related proteins in regulating adult hippocampal neurogenesis (AHN); specifically, DNMT1, DNMT3A, and ten-eleven translocation methylcytosine dioxygenase 1 (TET1) control proliferation and maintenance of NSCs and the differentiation, maturation, and survival of adult-born neurons (Jobe & Zhao, 2017). Specifically, DNMT1 functions to maintain DNA methylation patterns during proliferation of neurons, thereby preserving the identity of specific neuronal subtypes (Noguchi et al., 2015). DNMT3A is important in early stages of neurogenesis, functioning to establish methylation patterns in developing neurons, which in turn influences the expression of genes responsible for neuronal differentiation and maturation (Wu et al., 2010). TET-1 functions to remove or modify DNA methylation patterns through demethylation, which is required for dynamic changes in gene expression, and thus is necessary for neuronal differentiation and integration into existing neural circuits (Zhang et al., 2013). Although the specific roles of these proteins in rodents have been clarified, there continues to be interest in global DNA methylation patterns in the adult brain. This area requires further investigation, especially in non-rodent models.

The patterning of global DNA methylation appears to be regionally specific in the adult rodent hippocampus, where different levels of methylation across regions can be attributed to the role of the hippocampus in learning and memory (Brown et al., 2009). In other words, regional differences in DNA methylation in the hippocampus are attributed

to epigenetic regulation of specific genes that are associated with learning and memory in neurons. Therefore, DNA methylation regulates neural activities and memory formation by regulating the expression of genes in neurons. However, the relationship between neurogenesis and regional, global-DNA methylation remains elusive, partly due to the scarcity of neurogenic regions in the adult mammalian brain.

Like mammals, neurogenesis in birds is involved with and can be necessary for behavioural plasticity, specifically in learning and memory (Pytte, 2016). But unlike mammals, adult birds exhibit high levels of neurogenesis throughout their brains (Goldman, 1999). For example, in food-storing species like the black-capped chickadee (*Poecile atricapillus*), adult hippocampal neurogenesis is necessary for successful retrieval of food caches—a behaviour requiring spatial memory—and the rate of neurogenesis in the hippocampus varies with environmental and behavioural input (Hall, Delaney, & Sherry, 2014; Guitar & Sherry, 2018; Chancellor et al., 2011). Neurogenesis is also related to vocal learning and performance, which in some species, like the European starling, varies across season (Sartor, 2004). The seasonal changes in environment and behaviour also modulates rates of and is accompanied by changes in neurogenesis in the songbird vocal system—a series of nuclei involved in the learning and production of bird song and other types of vocalizations. And in laboratory settings, neurogenesis in auditory perceptual brain regions is associated with auditory discrimination learning in zebra finches (Newman, 2022). To date, research investigating the possible role of DNA methylation as a regulatory mechanism of neurogenesis in songbirds is minimal, however, it does appear to be involved in some aspects of behavioural plasticity. For example, in the great tit (*Parus major*), methylation and

demethylation of the promoter region of transcription factor NR5A1, which is involved in sexual development and reproduction, may regulate circannual breeding behaviour (Lindner, 2021). It is reasonable to speculate that changing patterns of DNA methylation between more and less neurogenic regions of the songbird brain may regulate differential rates of neurogenesis that underlie behavioural plasticity.

In the current study, we sought to establish a histological method for visualizing DNA methylation in the songbird brain and quantify any regional differences between species and sexes, and among regions. To do this, we adapted the protocol for labeling DNA methylation in rodents used by Brown et al. (2008) for use with songbird. We used immunohistochemistry to label 5-mC in the brains of three songbird species: black-capped chickadees, European starlings (*Sturnus vulgaris*), and zebra finches (*Taeniopygia castanotis*) that had been subjects in separate studies. Further, we assessed regional differences in global DNA methylation patterns within birds. Findings from this study will be useful alongside other epigenetic tools to establish region-specific patterns of DNA methylation in studies investigating the role of epigenetic mechanisms in regulating neurogenesis.

2.2 Experiment 1: Black-capped Chickadees

The purpose of this experiment was to adapt and verify an immunohistochemistry (IHC) protocol used in mouse tissue to detect global DNA methylation in neurons in avian tissue. The protocol was originally developed by Brown et al. (2008), who used the protocol to detect regional differences in neuronal methylation in mouse hippocampus.

2.3 Method

2.3.1 Subjects

Eight male and nine female black-capped chickadees were captured between May 2013 and February 2014 in the Halifax Regional Municipality (Nova Scotia, Canada) under permit from Canadian Wildlife Service (CWS; ST2779). Chickadee vocalizations were played from a field speaker to attract birds to our capture sites. We used potter traps baited with sunflower seeds to capture birds when their natural food sources were scarce (i.e., late fall and throughout winter), and when their food sources were abundant (i.e., late winter and spring) we used mist nets. Upon capture, birds' outer rectrices were examined to determine whether they were adults (see Pyle, 1997 for a description of method); birds determined to be less than one-year-old were released immediately. For birds determined to be more than one year old, we measured their weight, wing chord and tail length, and then placed them in cloth bags for transport to our research facilities.

2.3.2 Housing

Upon arrival at our facilities, birds were housed individually in galvanized steel mesh cages measuring 91 cm x 41 cm x 46 cm. Cages contained two wooden perches, a natural wood branch, a plastic bird bath, a swing, and food and water cups. Birds had *ad libitum* access to water, grit, and husked sunflower seeds mixed with Mazuri Small Passerine diet feed. Mealworms and unhusked sunflower seeds were placed on top of the feed daily. Housing rooms were maintained at an ambient temperature of 18°C. Birds captured in spring were maintained on a light schedule reflecting the natural light schedule for that time of year. Birds captured during winter were initially maintained on a

natural light schedule, but were acclimated to a spring light schedule, incrementally, over a period of 6-8 weeks.

2.3.3 Behavioural experiment – Operant conditioning

For the experiment, birds were transferred to individual, soundproof chambers measuring 65 cm x 46 cm x 42 cm. Each chamber contained a 37 cm x 31 cm x 24 cm birdcage fitted with an operant discrimination feeder, which could be accessed via an entry point on the rear, left side of the cage. Cages contained two wooden perches, a grit cup, and a water cup, and were lighted by a 9 W fluorescent bulb. The perch nearest the feeder was equipped with an infrared sensor, which when triggered, would initiate a trial. The feeder was also fitted with an infrared sensor that monitored when a bird entered the feeder. The experiment required birds to discriminate between stimuli in a set of altered fee-bee calls. When birds responded to the rewarded stimuli, they were given access to food via a motorized food hopper. When they responded to non-rewarded tones, the light was extinguished for 30 s. Detailed methods and results of the behavioural experiment are reported in Roach, Mennill, and Phillmore (2017).

2.3.4 Perfusion and Histology

After the behavioural experiment, birds were returned to their individual cages with *ad libitum* diet for three to four days before they were killed. Birds were given a lethal dose of 1:1 Xylazine:Euthanyl solution and perfused transcardially with PBS, followed by 4% paraformaldehyde. Birds' brains were harvested and stored in 4% paraformaldehyde for two days before being moved to a 30% sucrose in PBS solution until saturation. Once saturated, the brains were removed from the sucrose solution, frozen of pulverized dry ice, and stored at -80°C until further processing. Birds' gonads

were examined after the perfusion to determine their sex. Brains were sectioned coronally at a thickness of 40 μm (-17°C; 12° blade angle). Sections were separated into four series collected at 160 μm intervals and stored at -20°C in a 30% sucrose and 30% ethylene glycol in PBS solution.

2.3.5 Immunohistochemistry and Microscopy

We adhered to the protocol described in Brown et al. (2008) whenever possible, but there were several notable changes: 1) we used formalin-fixed brain tissue, whereas the original authors used flash-frozen tissue; 2) we excluded the mouse-on-mouse (MOM) blocking reagent they used as our primary tissue was avian, and 3) we performed all steps on free-floating sections of tissue, whereas Brown et al. performed IHC on tissue that had been thaw-mounted onto gelatin slides. All steps were performed at room temperature unless stated otherwise. For the first test run, we washed tissue five times for 5 min in PBS with agitation before incubating in 4% paraformaldehyde for 25 min. Tissue was washed three more times and transferred to 50% ethanol for 30 min at room temperature. The paraformaldehyde and ethanol treatments were omitted in a subset of tissue to qualitatively assess any differences in staining attributable to these steps. This step preceded three more washes, followed by a 30 min incubation in 2N HCl at 37˚C to expose genomic DNA. The tissue was then washed and neutralized in a 0.1M borate buffer for 10 min, followed by three more washes in PBS.

Tissue was blocked by incubating in the normal serums of the host animal of the secondary antibodies: normal horse and normal goat serums mixed with 0.3% PBS/T at 5% each for 1 h. After blocking, tissue was simultaneously incubated in rabbit anti-NeuN (Abcam ab104225) and mouse anti-5-mC (EpiGentek 33D3) antibodies at concentrations

of 1:500 and 1:250, respectively. Tissue was incubated in the primary antibodies overnight at 4˚C.

The following day, tissue was washed twice for 5 min before being incubated in secondary antibodies. Dylight 488 (Vector, Dl-2488) horse anti-mouse and Texas Red goat anti-rabbit (Vector, TI-1000) were prepared in low light at 1:400 in PBS. Tissue was incubated in the secondary antibodies for 1 h followed by 5 washes in PBS. Tissue was float mounted onto slides in low light and cover-slipped using VECTASHIELD Antifade Mounting Medium with DAPI (Vector, H-1200-10).

We captured images from the hippocampus using an Olympus DP80 camera mounted on an Olympus BX-51 microscope equipped with FITC, TxRed, and DAPI light filters. Images were coloured using the Combine Channels function and built-in fluorochrome presets in cellSens software (Olympus).

Based on qualitative analyses of images from this first test protocol, we omitted the paraformaldehyde and ethanol incubations in all subsequent runs, as these steps appeared to have no effect on staining quality (Figure 2.2). Due to a low signal:noise ratio of the labeling to background where overall the labeling was high and thus too high in the background, we reduced the concentration of primary antibodies to 1:1000 and 1:500 for NeuN and 5-mC, respectively. We also added 1% normal serums to the primary antibody solution for incubation to reduce non-specific staining. These adjustments were successful in reducing background noise and increasing the signal:noise ratio, so subsequent runs adhered to these changes. See Figure 2.3 for representative images of tissue from the first run (A) and subsequent runs (B).

2.3.6 Imaging and Quantification Using Optimized Protocol

We captured images from NCM, CMM, and HVC using the zebra finch brain atlas as a reference for locating each region (Nixdorf-Bergweiler & Bischof, 2007). We also used images from Matragrano et al. (2013), who identified these regions via staining for ZENK, a marker typically associated with measures of neural activity. We developed an imaging strategy to estimate their location by moving the section within the field of view from rostral to caudal and identifying cytoarchitectonic landmarks to approximate the location our regions of interest. We identified an area where the lamina mesopallialis (LaM), and the lamino pallio-subpallialis (LPS) converge near the midline as a key landmark to locate the regions of interest. Figure 2.4A shows a schematic diagram of a coronal brain section with the regions of interest identified.

Images were captured from three sections of tissue, resulting in three images per region, per hemisphere, per bird. We sought to identify and quantify cells that were co-labeled with 5-mC and NeuN; however, we were unable to definitively identify cell boundaries when fluorescent channels were combined (Figure 2.5). Therefore, we used three methods to quantify the images: cell counts (5-mC and NeuN), whole-image stain intensity (5-mC only), and cell stain intensity (5-mC only). For cell counts, NeuN-immunoreactive (ir) and 5-mC-ir cells were counted exhaustively in all images and averaged within region and hemisphere. For whole-image stain intensity, we used ImageJ to measure the stain intensity of the entire image (mean grey area) and three regions of the image background—i.e.., where no stain appeared. The three regions were averaged to estimate the image background stain intensity, which was subtracted from the whole-image stain intensity to generate our image label intensity value. We also used ImageJ to

measure the stain intensity of five clearly defined cells within each image. Cell stain intensity was calculated by subtracting the mean background stain intensity from the average stain intensity of the five cells. For all measures, if a section was missing from one hemisphere, the value from the other hemisphere was imputed.

2.4 Results

Tests yielding a *p* value of 0.05 or less are considered statistically significant. Tests yielding a *p* value between 0.05-0.1 are considered statistical trends.

Our first aim was to test for hemispheric and regional differences in 5-mC count, image stain intensity, and cell stain intensity. We also tested for regional and hemispheric differences in NeuN cell counts. Sex was not included as an independent factor in the analyses due to a labeling error which precluded us from linking data to the brain tissue. We ran paired t-tests to assess hemispheric differences in each region for each dependent variable. For 5-mC cell counts (Figure 2.6A) and 5-mC cell stain intensity (D), there were no hemispheric differences for any region. For 5-mC image stain intensity, we found that birds had more stain intensity in the left hemisphere of HVC than in the right ($t(13)=-2.247$, $p=0.043$) (Figure 2.6C). There were no other hemispheric differences for 5-mC image stain intensity. For NeuN count, we found a trend that birds had more NeuN-ir cells in the right hemisphere of HVC than in the left ($t(13)=2.090$, $p=0.057$) (Figure 6B).

Next, we assessed regional differences in each of our dependent variables. For 5-mC cell counts, we found that birds had more 5-mC-ir cells in CMM than in HVC, $t(13)=6.607$, $p<0.001$. There were no other regional differences in 5-mC cell counts. For NeuN-ir cell counts, we found that birds had more NeuN-ir cells in NCM than in HVC,

$t(12)=2.275$, $p=0.042$. For 5-mC-ir image stain intensity, we found that stain intensity was greater in CMM than in HVC, $t(13)=3.201$, $p=0.007$. There were no other regional differences in 5-mC image stain intensity. For 5-mC cell stain intensity, we found a trend that there was greater stain intensity in CMM than in NCM, $t(13)=1.977$, $p=0.07$. There were no other regional differences for 5-mC sell stain intensity.

2.5 Discussion – Experiment 1

The primary goal of this study was to adapt the immunohistochemistry protocol for visualizing 5-mC in rodents to avian tissue. We successfully adapted the protocol to chickadee tissue, but the quality of the visualization was poorer than reported by Brown et al. (2008). In that study, 5-mC staining exhibited sharp cell boundaries, low background, and the appearance of 5mC-ir puncta within cells. Comparatively, 5-mC staining in the current study suffered from a high level of background noise, which made cell boundaries less distinct and obscured the puncta that were observed in rodent tissue. The difference in the quality of staining likely limited our ability to detect differences in stain intensity at the image and cell level. The background noise also prevented us from using co-labeling to limit our analysis of 5-mC stain intensity to neurons (i.e., NeuN-ir cells), because the merging of two already noisy images further obscured the detection of cell boundaries.

There are some notable differences in the preparation of tissue in the current study compared to Brown et al. (2008). First, the tissue in their study was collected via rapid decapitation followed by flash freezing. The tissue in our study was collected after perfusion with heparinized PBS and fixation with 4% paraformaldehyde, the latter of which can lead to DNA degradation, which could account for the relatively low-level of

5-mC puncta observed in our tissue. Future studies investigating DNA methylation in avian brain tissue should compare visualization results from fresh-frozen and fixed tissue to further assess the effect of this methodological difference. Second, tissue in Brown et al. (2008) was sectioned at 16 μm and thaw mounted onto slides before immunohistochemistry. In comparison, our tissue was sectioned at 40 μm and processed as free-floating sections, which were then mounted onto slides. Using thinner section could enhance penetration of substrate into the tissue; however, tissue in the current study was processed free-floating, meaning it was exposed to substrate from both sides, as opposed to only the exposed side of the tissue as with thaw mounted tissues perhaps compensating for using thicker tissue. Finally, due to the host species of the primary 5-mC antibody being mouse, the authors used a Mouse-on-Mouse (MOM) blocking reagent to block endogenous immunoglobulins. The blocking kit produces clear, specific staining that seemed to outperform what could be achieved using normal serums for blocking in our tissue.

Nonetheless, we were able to measure 5-mC-ir cell counts and stain intensity across brain regions and hemispheres in chickadees, which was the secondary purpose of this study. We found that the number of 5-mC-ir cells was consistent across brain regions, suggesting that unlike in mammalian hippocampal tissue, differences in global 5-mC methylation at the cell level may not present in avian tissue. Qualitatively, however, there appeared to be more 5-mC-ir cells in NCM and CMM than in HVC, but more between-subject variability in 5-mC-ir cell counts in HVC. In HVC, cell density is in influenced by several factors, not limited to sex, season, and captivity (Gahr et al., 2008; Tramontin & Brenowitz, 1999). In this study, subjects' sex was not recorded when their brain tissue

was collected, so we did not use sex as an independent measure. Since we did not account for sex in the current study, it is not surprising that there was greater variability in 5-mC-ir cell counts HVC. However, since all subjects were held under the same light:dark cycle, we would not expect any effects as a result of short/longer daylengths. With regard to captivity, it is reasonable to anticipate a baseline change in cell density in HVC for all birds. However, the impact of captivity on global DNA methylation in the regions of interest remains unclear. Considering the extensive literature highlighting diverse factors influencing DNA methylation across different taxa, direct studies of the effects of captivity on global DNA methylation are warranted. In future studies, these factors should be accounted for in order to determine whether doing so allows for detection of regional effects in 5-mC-ir cell counts.

We found that stain intensity was greater in CMM than in HVC. This result aligns with the qualitative finding above, that CMM and NCM appeared to have more 5-mC-ir cells than HVC. Since there were more cells in these regions, it follows that the images from these regions would have greater overall stain intensity. However, when looking at 5-mC-ir cell-stain intensity, we found that cells in CMM have greater cell-stain intensity than cells in NCM. This finding is especially compelling because cell counts and whole image stain intensity were consistent across these regions. This finding suggests that DNA methylation affects cells in these regions differently. And given the related but differential roles of these regions, it seems plausible that methylation could give rise to the differences in function. Indeed, both CMM and NCM are involved in auditory memory and discrimination, with both regions being implicated in recognition of conspecific and heterospecific song. However, NCM's function appears to be experience-

independent, while CMM may be tuned more specially to learned auditory signals (see Louder et al., 2019 for review). It is not known whether the experiment, which required birds to discriminate between manipulated, conspecific stimuli, had any effect on the regional differences observed; however, given the duration of the experiment and the learning required, it could be that the learning caused differential methylation patterns in these regions that aided in the formation of auditory memories. To investigate this, DNA methylation could be measured in perceptual brain regions of birds subjected to auditory discrimination and compared to a yoked control group who hear the acoustic stimuli but are not required to learn the discrimination rule (see exp 2 below). In addition to 5-mC staining, IEG co-labeling should be used for increased accuracy for detecting neuroanatomical markers. Further, staining for DNA methyltransferases could help determine whether these enzymes are up- or downregulated during learning, regardless of whether changes to methylation are detectable using 5-mC staining alone.

In this study, we successfully adapted the 5-mC protocol for use with avian tissue, but visualization could be improved. The results here also warrant further investigation of the involvement of DNA methylation in auditory discrimination in songbirds. The following study expanded the results here by looking specifically at birds subjected to an auditory discrimination operant task.

2.6 Experiment 2: Zebra Finches

The purpose of this experiment was to apply the protocol developed in Experiment 1 to tissue harvested from birds that had completed an auditory perceptual experiment using an operant discrimination task. For a thorough description of the experimental method, see Chapter 3. Here, we used a set of sectioned tissue from these birds and applied the

optimized immunohistochemistry protocol for 5-mC described above to assess labeling of DNA methylation in zebra finches, comparing males and females that had experienced different conditions in the behavioural experiment.

2.7 Method

2.7.1 Subjects

24 male and female zebra finches were purchased from Eastern Bird Supplies (Thetford Mines Sud, Quebec) and pet stores in the Halifax Regional Municipality (Nova Scotia). Prior to the experiment, birds were housed in pairs in 91 cm x 41 cm x 46 cm wire mesh cages that contained food, grit, and water cups, a plastic bird bath, wooden perches, a swing, and part of a tree branch. Birds had *ad libitum* access to Mazuri Small Passerine diet seed, water, and grit, and were given hard-boiled eggs two times per week. The light schedule was set to 8:00AM - 9:30PM (i.e., 13.5:10.5 light:dark). Animal husbandry adhered to guidelines set forth by the University Committee on Laboratory Animals (UCLA) of Dalhousie University.

2.7.2 Apparatus

During the operant experiment, birds were housed in 65 cm x 46 cm x 42 cm sound attenuating, ventilated chambers. Each chamber contained a 37 cm x 31 cm x 24 cm individual cage, with an access point to a motorized feeder at the left, rear side. The cage contained food, water, and grit cups, a cuttlefish bone, and two wooden perches. An infrared beam monitored whether the bird was perched outside the access point to the feeder. The feeder itself also had an infrared beam that monitored when a bird entered it. The rear, interior wall of the chamber had a red light used to signal food availability in the feeder. Finally, beside the feeder there was a speaker (Fostex FE108E), which

broadcast the stimuli at 70 dB, as measured equidistant to the nearest perch. Throughout the experiment, birds accessed food only via the feeder (see Figure 3.1 for a diagram of the operant apparatus).

2.7.3 Procedure

A more detailed description of the experimental procedure, including shaping, can be found in Chapter 3.

2.7.4 Discrimination Experiment

Birds in the experimental group (*n*=8) were trained to discriminate between two patterns of stimuli made of up song elements from recorded zebra finch song. Elements were arranged in both repeated element sequences – i.e., AAB, ABB, BAA, BBA – and non-repeated element sequences – i.e., ABA, BAB. Birds were rewarded with 1 s access to food when they responded to playback of non-repeated stimuli (S+) and punished with 30 s of darkness for responding to non-repeated sequences. Birds in the yoked group were not subjected to the discrimination task and fed *ad libitum* but heard playback stimuli in real time from the neighbouring cage of a bird in the experimental group. Birds in the control group were not subjected to the task and were not exposed to any playback or other auditory stimuli. The experimental period lasted three weeks, and on day 22, birds were killed, perfused, and fixed, and their brain were frozen for later processing.

2.7.5 Tissue Processing and Quantification

We processed tissue as described in Experiment 1 above, with one exception: tissue in this experiment was cut in the sagittal plane at 30 μm rather than the coronal plane in order to improve the accuracy with which we could identify and quantify cells in our regions of interest, NCM and CMM. In the current experiment, we took images from

the dorsal and ventral regions of NCM, one image from CMM, and we also took images from hippocampus (hp) and a control region, hyperpallium apicale (HA). Figure 2.4B shows a schematic drawing of the sagittal brain sections and regions of interest.

2.8 Results – Experiment 2

We ran a region x hemisphere x group (discrimination, yoked, control) x sex ANOVA for each dependent variable: 5-mC image stain intensity, NeuN image stain intensity, and 5-mC cell stain intensity. For tests that violated the assumption of sphericity, a Greenhouse-Geisser correction was applied.

For 5-mC image intensity there was a main effect of region, $F(5,60)=3.091$, $p=0.015$, $\eta p2=0.205$. We used t-tests to determine which regions differed and noted statistical differences in Figure 2.7A. There was a region x hemisphere x group interaction, $F(10,60)=3.433$, $p=0.001$, $\eta p2=0.364$ therefore we ran separate hemisphere x group ANOVAs for each region. For HVC, there was a main effect of group for the right hemisphere, $F(2,18)=3.678$, $p=0.046$. Pairwise comparisons showed that birds in the yoked group had greater 5-mC image stain intensity in the right hemisphere of HVC compared to birds in the experimental group ($p=0.017$) (Figure 2.8). Pairwise comparisons also showed that birds in the experimental group had more 5-mC stain intensity in the left hemisphere than in the right ($p=0.018$).

For NeuN image intensity, there was a main effect of region $F(5,75)=2.455$, $p=0.04$, $\eta p2=0.141$ (Figure 2.7C). We used t-tests to determine which regions differed and noted statistical differences in Figure 2.7C. There was also a region x hemisphere interaction ($F(5,75)=10.104$, $p<.001$, $\eta p2=0.402$), so we compared hemispheres within in each region; these differences are also noted in Figure 2.7C.

There was a region x sex x group interaction $F(10, 75)=4.122$, $p=0.003$, $\eta p2=0.355$. We performed separate group x region ANOVAs for male and female birds. There was no main effect of group for any region in male birds, but there was a main effect of group for female birds in HVC, $F(2,1)=43.683$, $p<0.001$, eta=.926. Post-hoc tests revealed that females in the control group had more NeuN-ir image intensity than females in the yoked ($p=0.017$) and experimental ($p<0.001$) groups (Figure 2.9).

There were no main effects or interactions for 5-mC cell stain intensity (Figure 2.7B).

2.9 Discussion

The purpose of Experiment 2 was to assess regional differences in global DNA methylation in auditory perceptual brain regions of male and female zebra finches subjected to an auditory discrimination task. Here, we applied the immunohistochemistry protocol adapted in Experiment 1 to tissue from birds used in Chapter 3 of this thesis. Unlike in Experiment 1 (chickadees), in Experiment 2 (zebra finches) we detected several regional differences in 5-mC stain intensity: CMM and HVC had the highest image stain intensity compared to NCM and Hp. However, in this experiment, we did not detect regional differences in 5-mC stain intensity at the cell level, suggesting that the differences in image stain intensity here could be due to regional differences in cell density, rather than cellular global DNA methylation.

Interestingly, finches in the experimental group had higher 5-mC image stain intensity in the right hemisphere of HVC than in the left, suggesting that the experimental manipulation may have affected lateralization of cells in this region. However, given that

this effect was not observed in 5-mC cell stain intensity, it may not be attributable to differences in global DNA methylation. Other studies have reported functional lateralization in HVC (Moorman et al., 2012; Cousillas et al., 2020); however, that measure of lateralization is reflected in the responsiveness of neurons in the regions investigated, rather than the number of neurons (Xie et al., 2022). In European starlings, the degree of lateralization in HVC is positively correlated with social integration – meaning that birds who have more preferred partners have a higher degree of lateralization when measuring the neuronal activity of familiar compared to unfamiliar stimuli. Further, the left hemisphere of HVC seems to be more important for discrimination than the right (Okanoya et al., 2001; see Moorman & Nicol, 2015 for a review of memory-related lateralization in songbirds), so if the experimental manipulation did in fact cause changes in DNA methylation, it could be that demethylation of DNA in the left hemisphere promoted formation of new auditory memories.

Alternatively, it could be that prolonged exposure to the discrimination task was sufficient for recruitment of new neurons to HVC, accounting for the greater 5-mC image stain intensity there. However, if that were the case, we would expect to find lateralization in NCM, too, which has been observed (Tsoi et al., 2014). Although Tsoi et al. (2014) found that degree of song learning in zebra finches exposed to an auditory discrimination task was positively correlated with asymmetry of new neuron density in NCM, the authors did not investigate HVC, so we cannot compare the present results to theirs. However, since our NeuN image stain intensity did not produce the same effect, lateralization here could be specific to cell types other than mature neurons—potentially,

new neurons as investigated in Tsoi et al. (2014). This prospect is investigated further in Chapter 3.

2.10 Experiment 3: European starlings

The purpose of this experiment was to apply the protocol developed in Experiment 1 to tissue harvested from another species, European starlings, captured throughout the year. For a thorough description of the experimental method, see Chapter 4. Here, we applied the optimized protocol to from Experiment 1 to determine whether 5-mC levels differ between male and female starlings captured in breeding season or non-breeding season.

2.11 Method

2.11.1 Subjects

Sixteen male (*n*=10) and female European starlings (*Sturnus vulgaris*) were captured in either May (*n*=8) or December 2018 on campus at Dalhousie University in Halifax, Nova Scotia. We captured birds using potter traps baited with food, like Doritos, Hickory Sticks, and French fries. All procedures were approved by the University Committee on Laboratory Animals (UCLA; protocol #17-120).

2.11.2 Perfusion and Histology

Upon arrival to our facilities, birds were weighed and then killed via lethal injection of 1:1 Xylazine:Euthanyl. Birds were transcardially perfused with heparinized PBS followed by 4% paraformaldehyde. Brains were harvested and placed in paraformaldehyde for two days, followed by one day in 30% sucrose in PBS until saturated. The following day, brains were frozen on pulverized dry ice and stored at -80°C until further sectioning. Brains were sectioned in the coronal plane using a cryostat (-17°C; 12° blade angle) at a thickness of 50 μm. Gonadectomies were performed to

identify birds' sex and breeding condition and testes were measured using a Vernier caliper.

2.11.3 Tissue Processing and Quantification

We again processed tissue as described by the optimized immunohistochemistry protocol in Experiment 1. In the current experiment we took images from the dorsal and ventral regions of NCM, and we also took images from medial (HPm) and ventral (HPv) hippocampus and a control region, hyperpallium apicale (HA). See Figure 4C for a schematic drawing of the coronal sections and regions of interest. In the starling tissue, cell boundaries and 5-mC puncta were not readily observable in most of the tissue analyzed, which precluded us from analyzing 5-mC-ir using cell stain intensity as a dependent measure. See Figure 11 for a representative image of the starling tissue. Results for this experiment focus on whole image stain intensity.

2.12 Results

We ran a region x hemisphere x group (breeding, non-breeding) x sex ANOVA for each dependent variable: 5-mC image stain intensity and NeuN stain intensity. For tests that violated the assumption of sphericity, a Greenhouse-Geisser correction was applied. There were no main effects or interactions for image stain intensity for 5-mC (Figure 10A) or NeuN (B).

2.13 Discussion

We attempted to quantify seasonal changes in global neuronal DNA methylation in European starlings. Although the staining protocol worked to some degree, in that there was labeling of 5-mC, imaging of individual cells was not sharp enough to detect the puncta observed in the black-capped chickadee (Experiment 1) and zebra finch

(Experiment 2) tissue (see Figure 11). In addition, there were no differences in 5-mC-ir between sexes or season in starlings. Although we cannot be sure that the lack of difference in methylation (as measured by intensity) is due to lack of effects of sex and season, we also cannot be sure that there was an issue with the application of this immune protocol to starling tissue, or this starling tissue in particular. However, since 5-mC was detected, it is more likely the case that suboptimal tissue preparation affected our ability to produce precise results.

Like the tissue from other species used with this protocol, the starlings were perfused with heparinized PBS and 4% paraformaldehyde, but given their relatively larger mass, the birds required longer perfusions to clear the blood and become fixed. As mentioned above, the longer perfusion and fixation times could have affected DNA integrity. In future studies investigating global DNA methylation, I recommended post-fixing sectioned tissue for a short incubation period (~30 min) as described in Brown et al. (2008) to determine if DNA integrity can be improved.

2.14 General Discussion

The purpose of this study was to adapt a protocol for measuring global methylation levels in neuronal DNA in mammals for use with avian tissue and test it on three different songbird species with different backgrounds. Using the adapted protocol, we detected 5-mC in black-capped chickadee, zebra finch, and European starling brain tissue, but differences in how tissue was collected and fixed likely increased the variability in the labeling, and therefore somewhat limits our ability to compare in depth across our results and species.

Qualitatively, our adapted protocol produced superior images in zebra finch tissue compared to black-capped chickadees and European starlings. This is more likely due to differences in tissue preparation than the affinity of the 5-mC antibody to the tissue. Zebra finches are a common model species with which the researchers had more experience perfusing. The familiarity of the process made it easier to prepare the tissue quickly and effectively. Black-capped chickadees, being much smaller than zebra finches can be difficult to perfuse, and this was the researchers' first-time preparing starling tissue, a species several times the size of the zebra finch. Regardless of species, brain tissue can be removed more promptly and processed more consistently across individual subjects if fixation is done after harvesting the tissue. There is some benefit to fixing the tissue, specifically to maintain tissue integrity throughout the immunohistochemistry process, however, future studies should investigate the quality of staining in flash frozen, unfixed tissue. If better results are produced, performing immunohistochemistry on slide could help preserve tissue integrity compared to running immunohistochemistry on free-floating, unfixed tissue. In any case, perfusing animals and processing tissue is a highly technical skill that requires proper training and extensive practice to become proficient. Increasing the sample size to allow researchers to "practice" perfusing tissue to ensure some level of proficiency when working with experimental tissue is advised, though using all intact tissue in further processing and analyses is also imperative.

In chickadees, we detected a difference in 5-mC cell stain intensity between NCM and CMM. This finding is important because it validates the idea that regional differences in global DNA methylation at the cell level are detectable in avian brain tissue, as they are in mammalian tissue. NCM and CMM are related regions in that they are both

important for auditory processing but are functionally dissociated in that NCM is responsible for formation of long-term memories of conspecific song and CMM is primarily responsible for discrimination and categorization of auditory stimuli. Many studies have revealed their propensity to undergo plastic changes is coincidental to learning and memory formation (Mello, Nottebohm, & Clayton, 1995; Tsoi et al., 2014; Newman, 2022). The current finding suggests that these changes could be regulated, at least in part, by DNA methylation. To further validate this idea, future studies should simultaneously investigate the presence of methyltransferases to help determine whether differences in methylation are consequent to experience. And further, if the clarity of immunohistochemistry for 5-mc can be improved, such as by using flash-frozen, unfixed tissue, future studies should co-label with neuronal makers like Ki-67, DCX, and NeuN to help elucidate which aspects of neuroplasticity are being regulated by DNA methylation. However, because we were unable to sufficiently co-label the 5-mC cells, our interpretation of this finding is limited.

Given the role of DNA methylation and demethylation in the learning and memory, a promising area for future research would be to investigate methylation patterns in the hippocampus of spatial memory specialists, like the black-capped chickadee. Specifically, it would be interesting to investigate this phenomenon from a seasonal perspective, comparing global DNA methylation levels of birds in the fall and winter months when birds are storing food and forming memories of spatial locations to spring and summer months when food is abundant. Additionally, investigating DNA methylation in free-flying birds would be insightful, as it would help mitigate any potential confounding effects of captivity, which have been reported in spatial specialists.

Given the regional differences in 5-mC levels detected in our study, black-capped chickadees represent an ideal model for investigating the environmental influences on DNA methylation within an ecologically relevant context. These studies would enhance our understanding of the adaptive significance of DNA methylation in relation to cognitive processes in songbirds.

In conclusion, we successfully adapted the immunohistochemistry protocol for visualizing 5-mC in avian tissue, albeit with reduced visualization quality compared to the seminar study. The differences in staining quality likely limited our ability to detect precise variations in stain intensity at the image and cell level. Nevertheless, we measured 5-mC-ir cell counts and stain intensity across brain regions in black-capped chickadees, providing insights into global DNA methylation in avian brain tissue. The results demonstrated consistent 5-mC-ir cell counts across brain regions, suggesting that differences in global DNA methylation may be less pronounced in avian tissue than in mammalian hippocampal tissue. However, distinct variations in stain intensity were observed in CMM and NCM, regions associated with auditory memory and discrimination, implying a potential role of DNA methylation in modulating cell function in these areas. Future research should investigate the impact of captivity, sex, and season on global DNA methylation, as well as the involvement of DNA methylation in auditory discrimination and learning processes. Additionally, refining staining protocols and co-labeling techniques can offer more accurate and comprehensive insights into the role of DNA methylation in avian brain function. Overall, this study establishes the foundation for further investigations into DNA methylation in avian species and its implications for behaviour and cognition.

2.15 References

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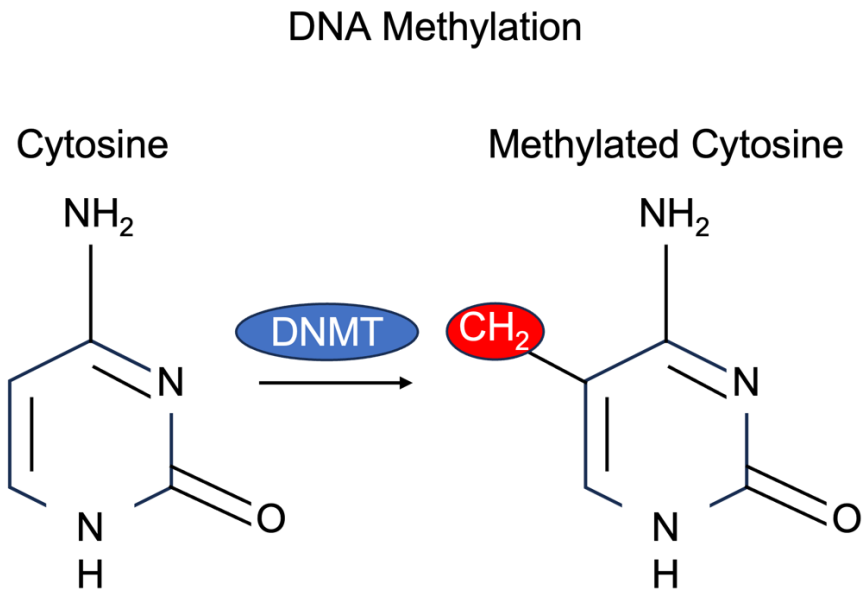

Figure 2.1. DNA methylation, which involves the addition of a methyl group to the DNA nucleobase, cytosine, via DNA methyltransferases (DNMTs). From Day and Sweatt, 2010.

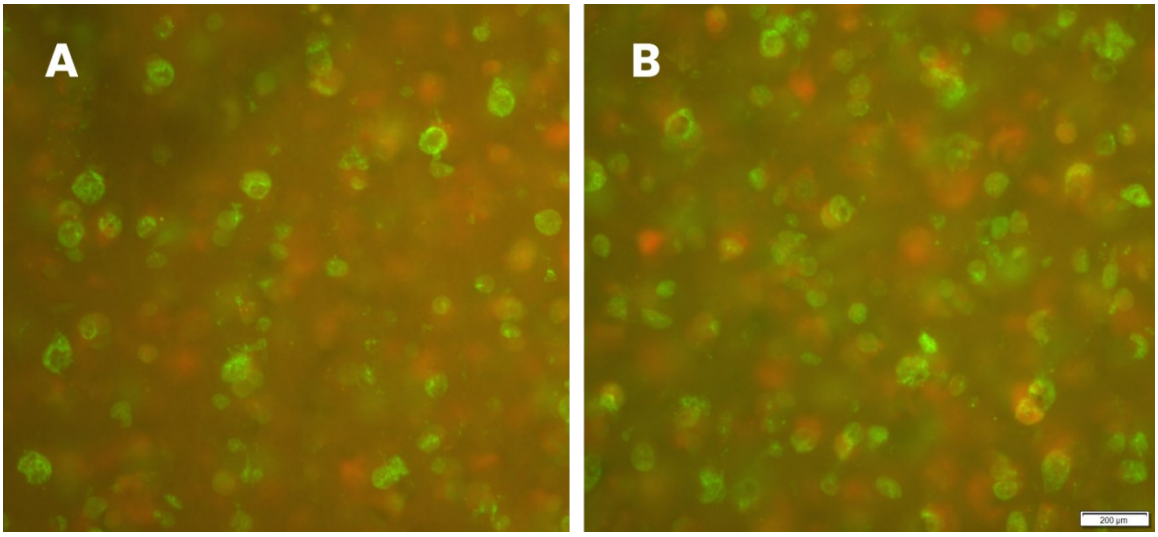

Figure 2.2. Representative examples of black-capped chickadee hippocampal tissue stained for NeuN (1:500; red) and 5mC (1:250; green) with alcohol and 4% paraformaldehyde fixation (A) and without (B). Treatment of tissue with alcohol and paraformaldehyde appeared to have no qualitative on the previously fixed tissue, though in both cases, tissue had a high degree of background staining. Images captured at 40x magnification.

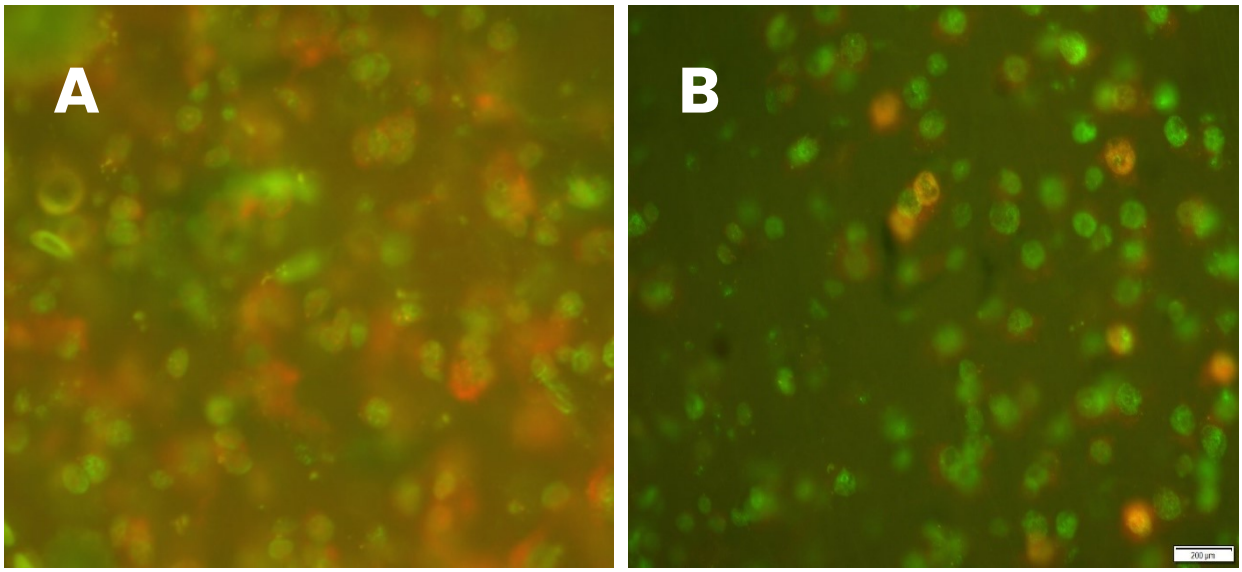

Figure 2.3. Representative examples of black-capped chickadee hippocampal tissue stained for NeuN (red) and 5mC (green) in the first (A) and subsequent (B) immunohistochemistry runs, captured at 40x magnification. In the first run, primary antibodies were run at 1:500 and 1:250 for NeuN and 5-mC, respectively. In subsequent runs, they were reduced to 1:1000 and 1:500, respectively. In the first run, primary antibodies were not incubated with normal serums, but in subsequent runs, primary antibody solutions contained 1% normal serums to reduce non-specific staining.

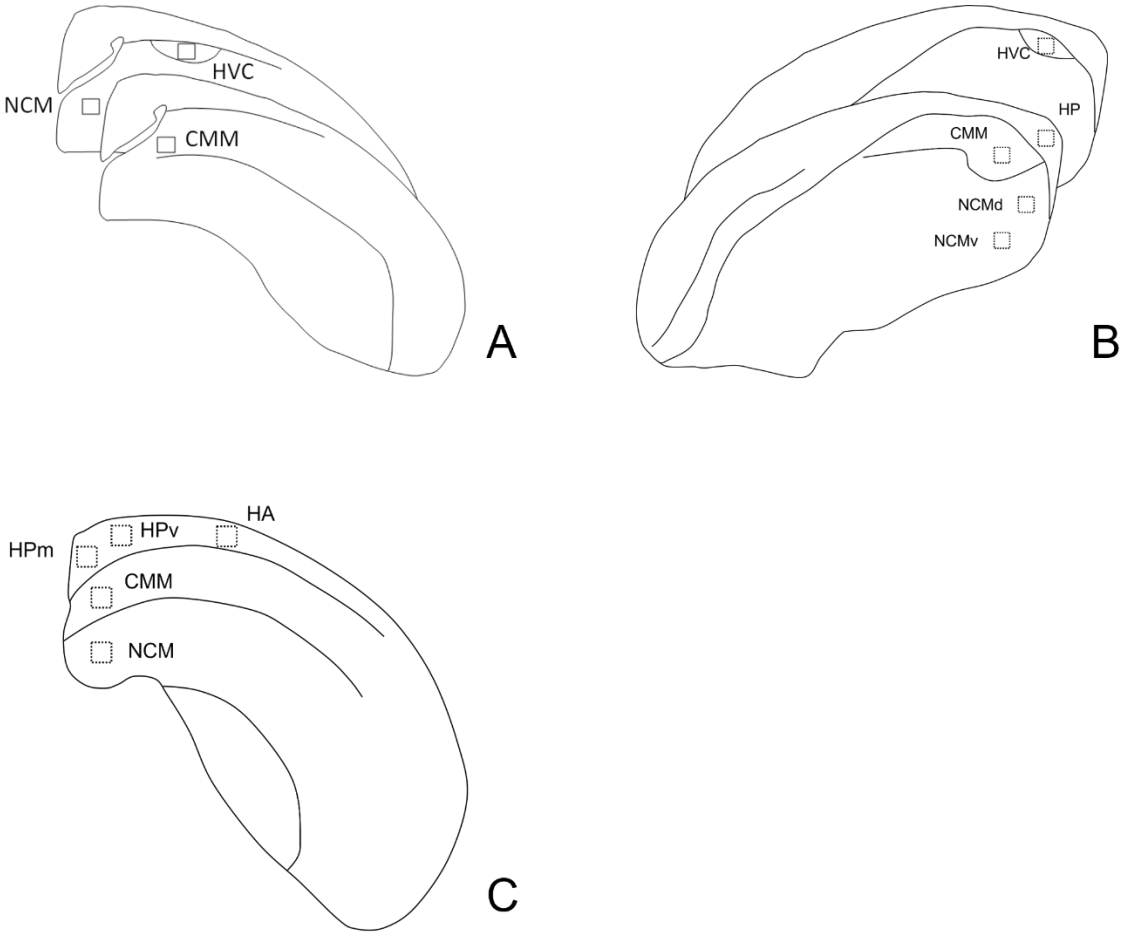

Figure 2.4. Schematic diagrams of sampling regions for each species. Panel A shows sampling areas within the CMM, NCM, and HVC in coronal sections of black-capped chickadee brain tissue used in Experiment 1. Panel B shows sampling areas within the CMM, NCMd, NCMv, HVC, and HP in sagittal sections of zebra finch brain tissue used in Experiment 2. Panel C shows sampling areas within the NCM, CMM, HP, and HA in coronal sections of European starling brain tissue used in Experiment 3. Interpreted from Nixdorf-Bergweiler & Bischof (2007).

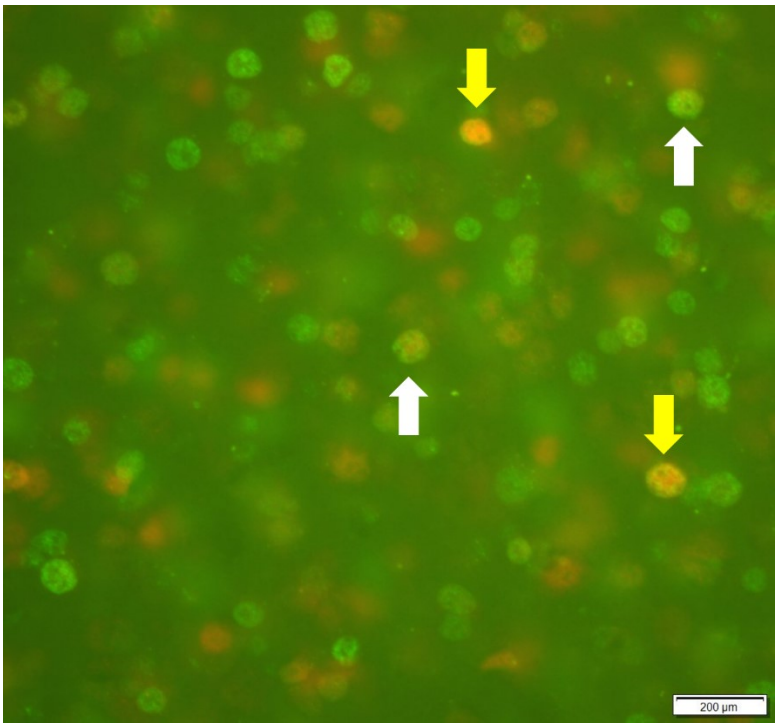

Figure 2.5. Co-labeling of 5-mC-ir (green) and NeuN-ir (red) cells in black-capped chickadee hippocampal tissue produced yellow colouring (yellow arrows). In many cases, however, we could not discern whether the yellow colouring was produced by co-labeling of a single cell or by a combination of cells not in focus (white arrows). Image captured at 40x magnification.

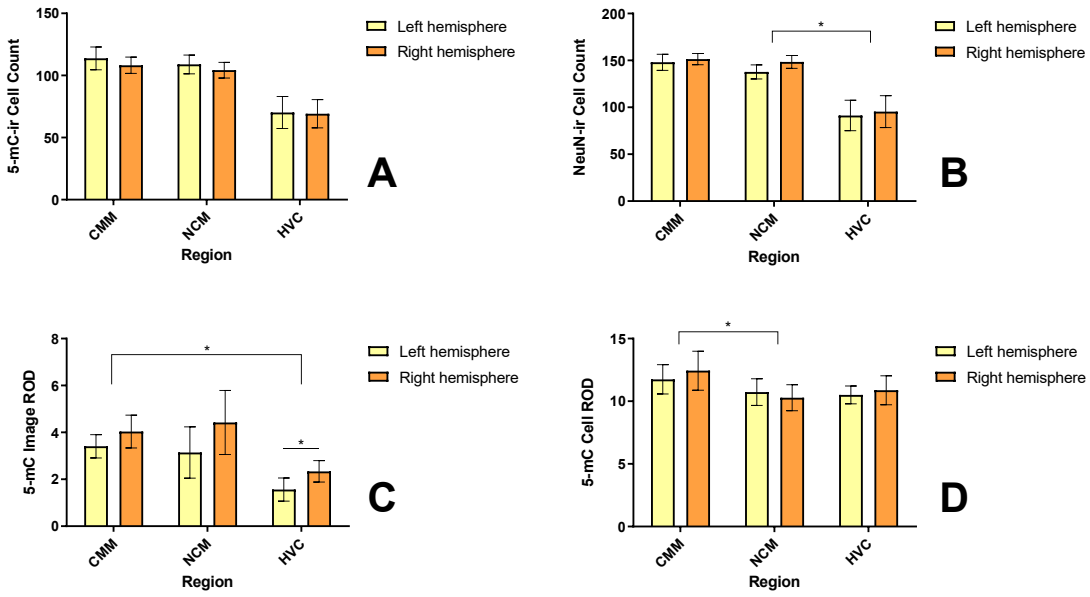

Figure 2.6. There were no regional or hemispheric differences in the number of 5-mC-ir cells (A). However, there were more NeuN-ir cells in NCM than in HVC. Chickadees had greater 5-mC image stain intensity in CMM than in HVC, and within HVC, there was greater 5-mC image stain intensity in the right than in the left hemisphere (C). Cells in CMM had greater 5-mC stain intensity than cells in NCM (D). Asterisks indicate significant effects at the $p < 0.05$ level.

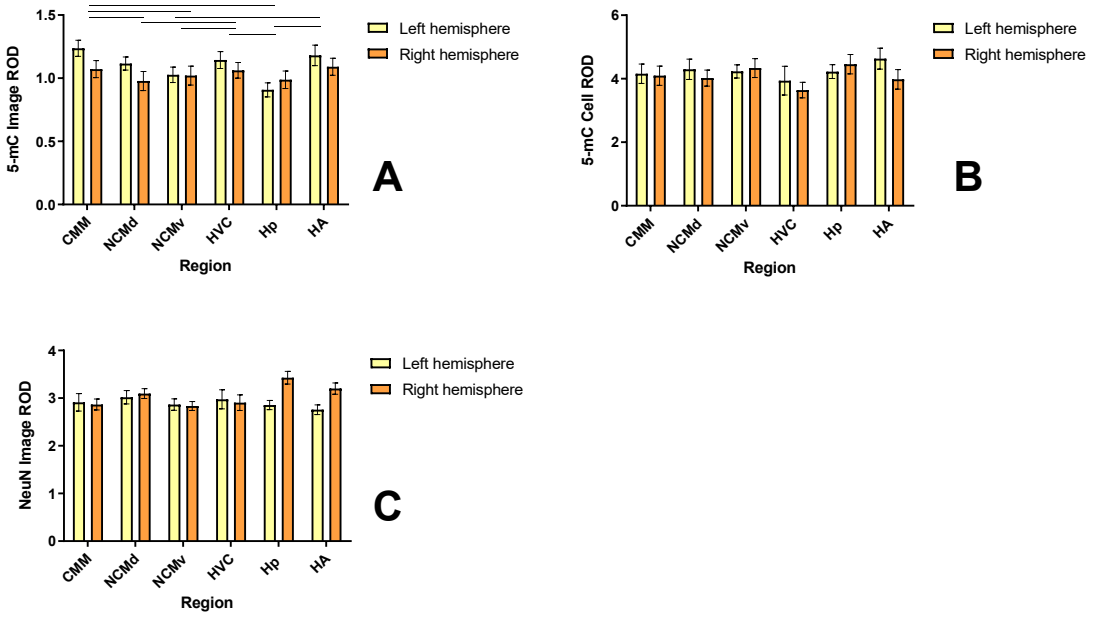

Figure 2.7. Regional levels of 5-mC image stain intensity (A), 5-mC cell stain intensity (B), and NeuN image stain intensity (C) in zebra finches, as measured by relative optical density (ROD) of staining. In panel A, horizontal lines indicate statistically significant differences in 5-mC image stain intensity between brain regions at the $p < 0.05$ level.

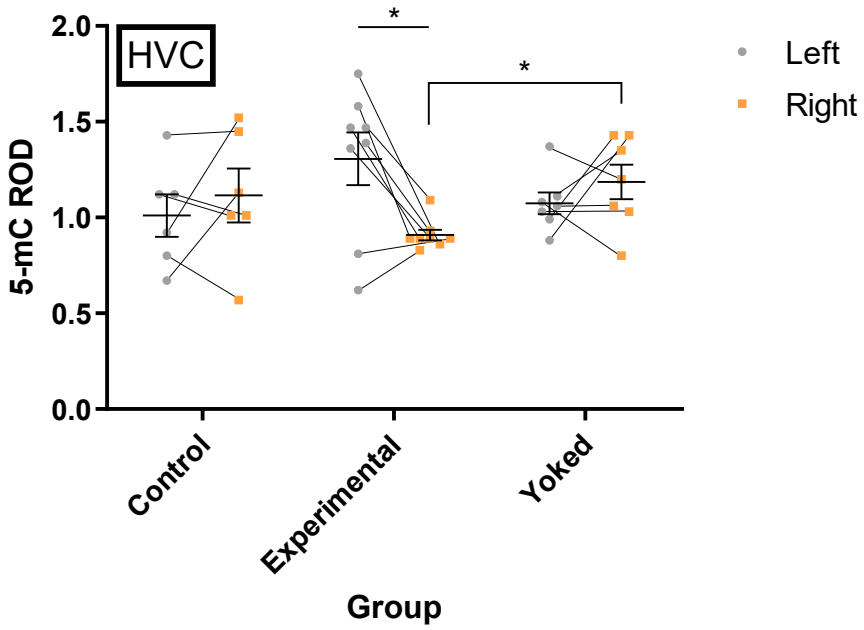

Figure 2.8. Zebra finches in the experimental group had greater relative optical density (ROD) of 5-mC image stain intensity in the left hemisphere of HVC than in the right. Zebra finches in the yoked group had higher 5-mC image stain intensity in the right hemisphere of HVC than finches in the experimental group. Asterisks indicate statistical differences at the $p < 0.05$ level.

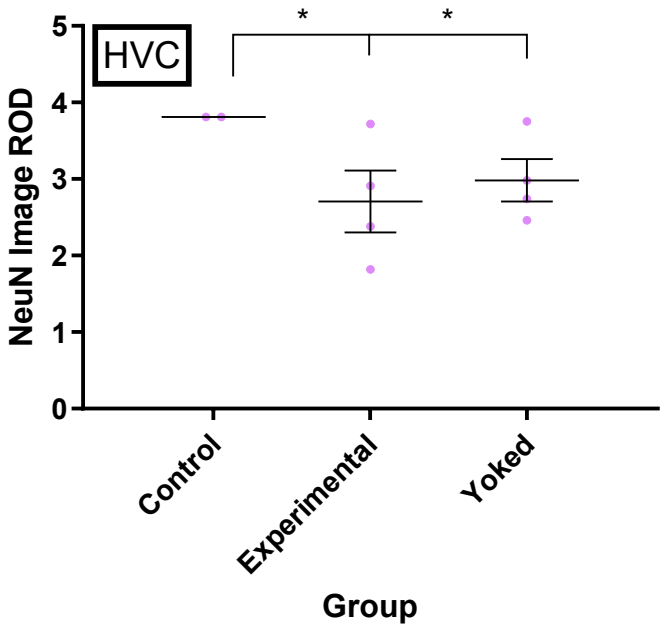

Figure 2.9. Female zebra finches in the control group had greater relative optical density (ROD) of NeuN image staining in HVC than female finches in the experimental group and in the yoked group. Asterisks indicate statistical differences at the $p < 0.05$ level.

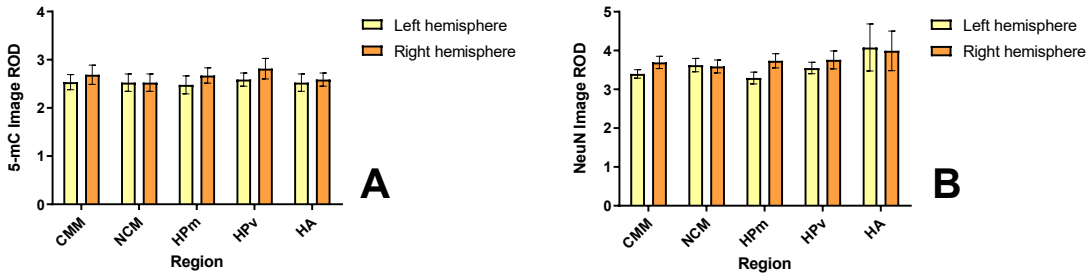

Figure 2.10. 5-mC image stain intensity (A) and NeuN image stain intensity across brain regions in European starlings, as measured by relative optical density (ROD) of fluorescent staining.

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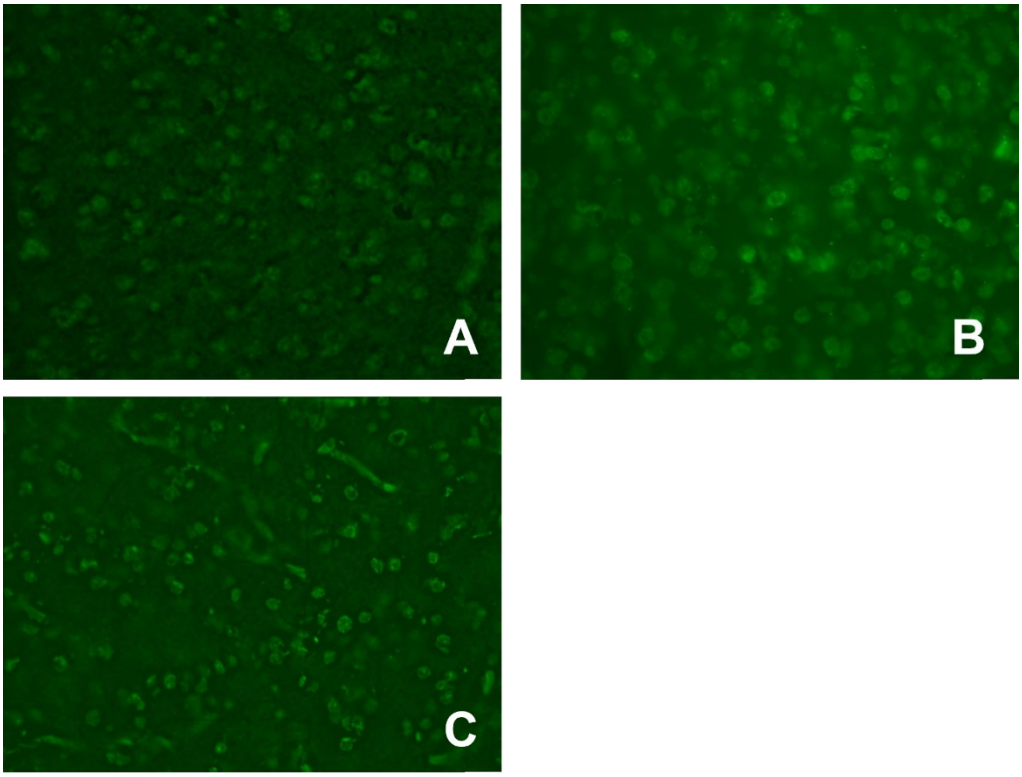

Figure 2.11. Sample images from CMM of European starling (panel A), black-capped chickadee (panel B), and zebra finch (panel C). 5-mC labeled cells in starling tissue (A) had poorly defined cell boundaries and lacked densely stained puncta observed in chickadee (B) and finch (C) tissue. The difference in stain quality may be attributable to differences in tissue preparation rather than true between-species differences in global DNA methylation. Images captured at 40x magnification.

CHAPTER 3: OPERANT DISCRIMINATION OF ACOUSTIC STIMULI DIFFERENTIALLY AFFECTS LATERALIZATION OF DOUBLECORTIN (DCX) EXPRESSION IN AUDITORY BRAIN REGIONS OF ADULT MALE AND FEMALE ZEBRA FINCHES

3.1 Introduction

In songbirds, vocal learning and production are controlled by the avian vocal system—a series of nuclei dedicated to the acquisition, production, and maintenance of vocalizations, including song (Kirn, 2010; Brenowitz et al., 1997). The vocal system includes two pathways related to vocalizing: the motor pathway, responsible for vocal production, and the anterior forebrain pathway, responsible for vocal learning. One nucleus, HVC (not an acronym), plays a crucial role in both pathways and has been implicated in vocal learning during development, as well as learning and maintenance throughout life (Alvarez-Buylla et al., 1992; Pytte et al., 2012). HVC also, as part of the auditory forebrain pathway, connects directly or indirectly to other regions including two responsible for storage of long-term auditory memories and discrimination of auditory stimuli: the caudomedial nidopallium (NCM) and caudomedial mesopallium (CMM). In zebra finches, NCM and CMM neurons selectively fire in response to conspecific songs and adapt to repeated exposures of auditory stimuli (Bell, Phan, & Vicario, 2014). In NCM, neurons respond differently to GO (rewarded) and NoGO (non-rewarded) stimuli, but novel stimuli elicit the greatest response (familiarity) (Bell, Phan, & Vicario, 2014). In contrast, in CMM, neurons respond more strongly to both GO and NoGo stimuli compared to novel stimuli (reinforcement predictive). Additionally, the rate of adaption of neurons in these regions are positively correlated with operant task performance, where fast learners have quicker adaptation of responses compared to slower learners.

These findings suggest that NCM and CMM have complementary but distinct roles in auditory tasks such as operant discrimination and that the dynamics of neuronal responses, as measured using electrophysiology, in these regions are linked to learning ability.

In addition to modifying neural responses, auditory learning and memory have been linked to neurogenesis in NCM. For instance, the quality of song learning in adult male zebra finches corresponds to increased hemispheric asymmetry of new neurons, indicating that new neurons are allocated according to the functional demands of the respective hemispheres (Tsoi et al., 2014). Additionally, female zebra finches who were exposed to a difficult acoustic discrimination task (but not an easy one) had more new neurons and a higher degree of hemispheric lateralization of those neurons in the NCM than yoked controls, providing further evidence that functional demands can influence new neuron survival in auditory brain regions (Newman, 2022). However, in the latter study, where birds had to discriminate between two songs, the difference in difficulty between the hard (song stimuli were similar) and easy (song stimuli less similar) tasks was not as distinct as the authors intended, as some overlap existed in the amount of time required for subjects to achieve the learning criterion. As a result, while a correlation between auditory learning and memory and neurogenesis in NCM is evident, it remains uncertain whether performing a difficult auditory discrimination task can increase recruitment and survival of new neurons beyond baseline levels in male and female birds. Furthermore, although CMM's role in acoustic stimulus discrimination has been established, it remains unclear whether neurogenesis contributes to task performance in this related auditory perceptual region.

Environmental factors can also influence neurogenesis in the vocal system and auditory brain regions of songbirds. One of these is social context (Lipkind et al., 2002; Adar & Lotem, 2008). For example, zebra finches housed in groups have more new neurons in HVC and NCM than birds housed in pairs or isolation (Lipkind et al., 2002). Similarly, male zebra finches housed in large groups after being switched from small groups showed increased neurogenesis in HVC and NCM compared to those housed with a single female after the same switch (Adar & Lotem, 2008). The extent to which experimental conditions increase cognitive demands can influence neurogenesis, with complex social changes leading to enhanced survival of new neurons and death of old ones, and simple changes enhancing the survival of old neurons. Overall, these findings suggest that social context can modulate neurogenesis in the avian vocal system and auditory regions, potentially contributing to changes in vocal behaviour in response to social cues. However, whether providing a cognitively demanding task can mitigate aspects of a simple environment has yet to be explored.

In the current study, we aim to further our understanding of how auditory discrimination and social isolation impact neurogenesis in HVC, NCM, and CMM by building on previous studies in three distinct ways. First, we employed a challenging discrimination task that required the birds to discriminate between acoustic stimuli for three weeks, a sufficient duration for new neurons to migrate and become functionally integrated. Second, we included a control group subjected to complete auditory isolation except for the birds' own vocalizations so we could assess the potential of the discrimination task not only to enhance neurogenesis above baseline levels but also its potential to rescue any supressing effects caused by isolation. Last, we used an

endogenous marker of neurogenesis that permits visualization of neurons at various stages of neurogenesis so we could distinguish between neuronal subtypes. To do this, we utilized a "grammar learning" task that involved distinguishing between two three-note sequence patterns derived from zebra finch songs: a repeating pattern (e.g., AAB) and an alternating pattern (e.g., ABA). After three weeks of task exposure, we sacrificed birds to analyze doublecortin-immunoreactive (DCX-ir) fusiform and multipolar cells in HVC, CMM, and NCM. Fusiform cells possess small, spindle-like processes, while multipolar clearly defined cell bodies, axons, and dendrites.

Our hypothesis was that birds that actively engaged in the discrimination task would have more DCX-ir cells in CMM and NCM than birds that were not actively engaged in the task (both passive listening and isolated) and that birds passively listening would have more DCX-ir cells in CMM and NCM than isolated birds.

In their study, van Heijningen et al., (2013) reported no sex differences in performance of the discrimination task, suggesting male and female birds used the same strategies to learn the task; thus, we did not expect to see sex difference in neurogenesis in the perceptual regions. We expected to see a positive correlation between hemispheric asymmetry in NCM and CMM and task performance, suggesting a role of neurogenesis to support the plasticity necessary for auditory discrimination in adult birds. And finally, we expect a greater degree of hemispheric lateralization in birds who performed the discrimination task than those who only heard the stimuli passively.

3.2 Materials and Methods

3.2.1 Subjects and Housing

Twenty-four male (*n*=12) and female zebra finches (*Taeniopygia castanotis*) were purchased from Eastern Bird Supplies (Thetford Mines Sud, Quebec) and pet stores in the Halifax Regional Municipality (Nova Scotia). Upon arrival to our facilities, birds were weighed and banded before being placed in 91 cm x 41 cm x 46 cm galvanized steel, mesh cages. Birds were housed in same-sex pairs, but some pairs were separated temporarily when behaving aggressively. Each cage contained several food and grit cups, a plastic bird bath, two wooden perches, a natural tree branch, and a swing. Birds had *ad libitum* access to Mazuri Small Passerine diet feed, water, and grit, and were given grated hard-boiled eggs twice weekly. Ambient temperature in the housing room was maintained at 24°C, with lights turning on at 8:00AM and off at 9:30PM (i.e., 13.5:10.5 light:dark). Animal husbandry adhered to guidelines set forth by the University Committee on Laboratory Animals (UCLA) of Dalhousie University.

3.2.2 Operant Apparatus

During the operant discrimination experiment, birds were transferred to a 65 cm x 46 cm x 42 cm sound attenuating, ventilated chamber. Each chamber contained a 37 cm x 31 cm x 24 cm individual bird cage, with an access point to a motorized feeder at the left, rear side. The cages contained a grit cup, cuttlefish bone, and two wooden perches, one on either side of the cage. The perch closest to the feeder was fitted with an infrared beam, which monitored whether the bird was perched outside the access point to the feeder. The feeder was also fitted with an infrared beam, which monitored when a bird entered the feeder. On the rear, interior wall of the feeder, there was a red light used to signal food

availability. Finally, beside the feeder there was a speaker (Fostex FE108E), which broadcast the stimuli at 70 dB, as measured equidistant to the nearest perch. Throughout the experiment, birds accessed food only via the feeder. See Figure 1 for a diagram of the operant apparatus.

3.2.3 Software

Each operant chamber was connected to a computer running HyperTerminal software to communicate between the PC and the chamber, and CDComm to play audio via a CD drive. Virtual drives for CD playback were created using Daemon Tools software.

3.2.4 Stimuli

Stimuli were based on those reported by (van Heijningen et al., 2013). Individual zebra finch song notes were derived from our own song library and the zebra finch song library from the Laboratory of Vocal Learning (http://ofer.sci.ccny.cuny.edu/song_database/zebra-finch-song-library-2015/view). Eight stimulus sets were created, each made up of two note types (i.e., curve, downslide, flat, high, stack, trill). Each set had a different pair of note types. Using Adobe Audition, the two note types were arranged in six combinations containing either a repeated note type (i.e., AAB, ABB, BAA, BBA) or alternating note type (i.e., ABA, BAB). Notes were sequenced with 40 ms of silence between each note and 50 ms of silence before and after the first and last note of the sequence, respectively, to eliminate acoustic artifacts. The resulting stimuli ranged in length from about 400 ms – 500 ms, depending on the duration of the individual notes (See Figure 2 for example stimuli). For the experiment, responses to alternating sequences (S+) were rewarded with food access for 1 s, while responses to repeated sequences (S-) were punished with lights out for 30 s. Rewarded S+ and S-

stimuli were played at random with equal probability, meaning the ABA and BAB stimuli were played at two times the frequency of the repeated stimuli. The pool of stimuli had two of each of those stimuli, and four of the repeating stimuli. Additionally, a 500 ms 1000-Hz sine wave tone was used during the shaping procedure.

3.2.5 Procedure

3.2.5.1 Shaping

Upon transfer to the operant chambers, birds could only access food via the feeder. During the first phase of shaping (Shape 1), the red light on the far wall of the feeder remained on. On the first day of Shape 1, the food hopper remained accessible and additional seed was scattered on top of its cover, which had holes through which the bird accessed the hopper. On the second day of Shape 1, food was removed from the hopper cover, so birds could only access food through its holes; again, the red light remained on. The following day, the second phase of shaping (Shape 2) began, which involved intermittent access to food indicated by the red light, with the duration of food access and inter-trial interval decreasing incrementally. Throughout this phase, when the red light turned on, birds would enter the feeder, and the hopper would ascend for the birds to eat from. From day one to day two of Shape 2, the feed time decreased from 5 s to 3 s, and then remained at 1 s for days three and four. Similarly, the inter-feed interval decreased from 120 s to 90 s over the first two days, and then remained at 60 s for days three and four. All birds met the 100 feeds per day criterion for each day of Shape 1 and 2.

The third phase (Shape 3) involved training the birds to perch on the perch nearest the feeder access point for an increasing duration, before being given access to food. Once the bird perched for the required amount of time, the red light would turn on,

indicating that the bird could enter the feeder to access the hopper. The required perch duration increased from 100 ms to 500 ms over the first two days, and then remained at 1 s for days four and five. The birds could initiate another trial after 500 ms. If birds did not meet the 100 feeds per day criterion, day four was repeated before moving on to the next phase, 'tone plus light' (TPL).

TPL involved pairing the red light with the sine wave tone. To initiate a trial, birds had to perch for 1 s, after which time the light came on, either with or without the tone. Birds were only rewarded when they responded to the tone and light pairing; response to light only resulted in the cage lights being extinguished for 30 s. Birds continued TPL until they reached a discrimination ratio (DR) of 90%. DR was calculated by dividing the number of responses to rewarded stimuli (S+) by the number of responses to S+ plus the number of responses to non-rewarded stimuli (S-).

Once birds met criterion for TPL, they moved onto the 'tone no light' (TNL) phase, which required birds to discriminate between the presence and absence of the tone, without the light stimulus. The trials and criterion for TNL were the same as those for TPL.

The final shaping stage trained birds to remain on the perch for the duration of the tone (i.e., 500 ms). If birds left the perch before the tone finished playing, they were punished with lights-out for 30 s. Similarly, birds also received this punishment if they entered the feeder after perching for 500 ms and no tone was played. The criterion for Zaps was the same as TPL and TNL; however, birds were also required to avoid punishment on 90% of the trials.

3.2.5.2 Discrimination Training Group

After shaping, birds in the experimental condition (n=8) moved onto the pattern discrimination phase for three weeks. The trials for discrimination were the same as the Zaps trials, expect the tone was replaced with rewarded tone sequences (e.g., ABA) and the no-tone trials were replaced with the non-rewarded sequences (e.g., AAB).

3.2.5.3 Response Measures and Analyses

The dependent measures for the discrimination task included final discrimination ratio (fDR) and highest discrimination ratio achieved (hDR). As previously mentioned, the DR was calculated by dividing the number of responses to S+ by the number of responses to S+ minus the number of responses to non-rewarded stimuli S-.

3.2.5.4 Yoked Group

After shaping, birds in the yoked group returned to the *ad libitum* diet described above. Audio playback produced by trials performed a bird in the experimental group was directly routed to a chamber containing a bird in the yoked group via a speaker attached to an experimental bird's computer. The yoked group birds heard stimulus playback from a paired experimental bird throughout the three-week period but did not engage in the discrimination task.

3.2.5.5 Control Group

Birds in the control group were also returned to the *ad libitum* diet described above after shaping. They did not hear any auditory stimuli after shaping.

3.2.6 Neural Measures

3.2.6.1 BrdU Injections

After the shaping phase, all birds were placed on free feed and given four injections of BrdU at 2 h intervals. The following day, experimental birds moved onto discrimination

trials, while control and yoked birds remained on free feed. Results of the BrdU-labeled tissue are not reported here.

3.2.6.2 Perfusion and Histology

After the 22nd day of the experimental phase, birds continued trials for at least 90 min before they were sacrificed. During the first 30 min of trials, we recorded the number of trials performed by each experimental bird. After 30 min, we turned off the lights and audio to each booth and waited 1 h before capturing birds for sacrifice. Birds were transferred in cloth bags to our perfusion suite, where they were weighed, then euthanized via lethal overdose of 1:1 Xylazene:Euthanyl solution. Birds were transcardially perfused with PBS followed by 4% paraformaldehyde in PBS. Brains were harvested and placed in 4% paraformaldehyde for two days, followed by one day in 30% sucrose in PBS, before being frozen in pulverized dry ice. Tissue was stored at -80°C until further processing.

3.2.6.3 Immunohistochemistry

Brains were sectioned on the sagittal plane (each hemisphere separately) at 40 μm using a cryostat (-17°C; 12° blade angle) and stored at -20°C in a 30% sucrose and 30% ethylene glycol in PBS solution until immunohistochemistry (IHC) was performed. Three series of tissue were collected at 120 μm intervals.

3.2.6.4 Doublecortin (DCX)

Tissue was washed five times in PBS to remove cryoprotectant solution and then incubated in H_2O_2 for 30 min. Tissue was then washed twice in PBS and blocked in 10% normal horse serum in 0.3% PBS/T for 1 h. The DCX (C-18) primary antibody (Santa Cruz, sc-8066) was prepared in 0.3% PBS/T at a concentration of 1:250. Tissue was

incubated in the primary antibody solution over night at 4˚C. The following day, tissue was washed twice in 0.1% PBS/T before being incubated for 1 h in biotinylated horse anti-goat secondary antibody (Vector, BA-9500) at 1:400 in 0.3% PBS/T. This was followed by three washes in 0.1% PBS/T. Tissue was then incubated in avidin-biotin horseradish-peroxidase (Vector, ABC Elite) at a concentration of 1:200 for 1 h, then washed twice in 0.1% PBS/T. We visualized tissue using SIGMAFAST DAB (Sigma-Aldrich) for 90 s, then washed it four times in PBS. Tissue was float mounted onto gelatin-coated slides and coverslipped using Permount.

3.2.6.5 Microscopy

Brightfield images were captured at 40x magnification using an Olympus DP80 camera, Olympus BX-51 microscope, and CellSens software. With reference to the zebra finch brain atlas (Nixdorf-Bergweiler & Bischof, 2007), we captured three images per hemisphere from within HVC, CMM and NCM, with the entirety of each taken within the boundaries of a given region. See Figure 3A for a schematic of the sampling locations.

3.2.6.8 Tissue Quantification

To quantify DCX-immunoreactive (DCX-ir) cells, we counted cells exhaustively in each image. Cells with clearly defined cell bodies, axons, and dendrites were considered differentiated multipolar neurons (Mp), whereas small cells with small spindle-like processes were considered fusiform (f) cells. For CMM, HVC, dorsal NCM (NCMd), and ventral NCM (NCMv), we averaged the number of Mp and f cells in each hemisphere across three images. Further, we used the ‘threshold’ function in ImageJ to quantify the area of the image that was DCX-ir (%Cov). Distinguishing between f and

Mp cells (Figure 3B) and measuring DCX-ir %Cov (Figure 3C) allowed us to estimate the migration, differentiation, and maturation of new neurons, respectively. Here, we use the term maturation to encompass cell morphological characteristics not accounted for by cell counts, like cell size and extend of dendritic arborization.

3.2.6.9 Statistical Analyses

We ran a region x hemisphere x group x sex ANOVA for each dependent variable. For tests that violated the assumption sphericity, a Greenhouse-Geisser correction was applied. Tests yielding a p value of 0.05 or less were considered statistically significant. Test yielding a p value between 0.05-0.1 were considered trends.

3.3 Results

For multipolar cells (Figure 4A), there was a significant main effect of region, $F(3,39)=10.50$, $p<0.001$, $\eta p2=0.45$. Simple main effects showed that there were more DCX-ir multipolar cells in NCMd than in CMM ($p<0.001$) and in NCMv ($p=0.002$). Further, there were more DCX-ir multipolar cells in NCMv than in CMM ($p=0.049$) and in HVC than in CMM ($p=0.02$). There were no other main effects for multipolar cells. Results from the ANOVA revealed a significant region x sex interaction; $F(3)=6.69$, $p=0.001$, $\eta^2 p=0.34$. A follow-up ANOVA revealed that female birds had more DCX-ir multipolar cells in HVC than males; $F(1)=11.82$, $p=0.003$, $\eta^2 p=0.41$ (Figure 4B). There were no other interactions for multipolar cells.

For fusiform cells (Figure 5A), there was a main effect of region, $F(3)=8.76$, $p<0.001$, $\eta^2 p=0.40$. Simple main effects showed that there were more DCX-ir fusiform cells in NCMv than in HVC ($p=0.001$), CMM ($p<0.001$), and NCMd ($p=0.002$). There was also a main effect of hemisphere, with birds having a greater number of DCX-ir

fusiform cells in their right hemisphere than in their left, $F(1)=8.89$, $p=0.01$, $\eta^2p=0.41$. The were no other main effects for fusiform cells, but there was a region x hemisphere interaction and a region x sex x group interaction, $F(3)=3.81$, $p=0.017$, $\eta^2p=0.23$; $F(6)=5.81$, $p<0.001$, $\eta^2p=0.47$. A sex x region ANOVA for each group revealed that male birds in the experimental group had more DCX-ir fusiform cells in CMM than females, $F(1)=14.93$, $p=0.008$, $\eta^2p=0.71$ (Figure 5B). There was also a trend suggesting that female birds in the experimental group had more DCX-ir fusiform cells in HVC than males, $F(1)=4.97$, $p=0.067$, $\eta^2p=0.45$.

The initial ANOVA also revealed a region x hemisphere x sex x group interaction, $F(5)=2.51$, $p=0.037$, $\eta^2p=0.28$. Follow-up t-tests revealed that male birds in the experimental group had more DCX-ir fusiform cells in the right hemisphere of CMM than in the left ($p=0.002$) (Figure 5C). T-tests also revealed trends suggesting that male birds in the experimental group had more DCX-ir fusiform cells in the right hemisphere of NCMv ($p=0.06$) than in the left and that females in the yoked group had more DCX-ir fusiform cells in the right hemisphere of HVC than in the left ($p=0.083$).

For DCX-ir %Cov (Figure 6), there was a main effect of region, $F(3)=9.51$, $p<0.001$, $\eta^2p=0.42$. Simple main effects showed that there was more DCX-ir %Cov in HVC than in CMM ($p<0.001$), NCMd ($p<0.009$), and NCMv ($p=0.033$). There was also greater DCX-ir %Cov in CMM than in NCMv ($p=0.044$) and in NCMv than in NCMd ($p=0.005$; Figure 6A). There were no other main effects for DCX-ir %Cov. The ANOVA revealed a region x hemisphere x group interaction, $F(6)=2.45$, $p=0.042$, $\eta^2p=0.27$. Birds in the yoked group, irrespective of sex, had more DCX-ir %Cov in the right hemisphere of CMM than in the left ($p=0.04$) (Figure 6B). A trend in the data suggests that birds in

the control group had more DCX-ir %Cov in the left hemisphere of NCMv (0.078) than in the right.

We ran correlation analyses between performance (*Figure 7*) and brain measures. These analyses revealed that there was a significant, strong correlation between the number of DCX-ir fusiform cells in the right hemisphere of NCMv and both best DR (r=0.73, p=0.039) and final DR (r=0.75, p=0.033) (Figure 8). Figure 7 shows the discrimination performance of individual male (top) and female subjects.

3.4 Discussion

The purposes of this study were (1) to investigate whether engaging birds in a challenging auditory discrimination task would enhance the rate of migration of new neurons (i.e., fusiform cell counts) to auditory brain regions and their subsequent differentiation (i.e., multipolar cell counts) and maturation (i.e., stain coverage), and (2) to investigate whether passive exposure to auditory stimuli would mitigate effects of isolation on those same measures. Contrary to our hypothesis, performing an auditory discrimination task or merely listening to another bird perform the same task did not categorically lead to a significant increase in the migration, differentiation, or maturation of new neurons, as compared to the control group. Nevertheless, we observed more nuanced impacts of sex, hemisphere, and region on neurogenesis within groups.

3.4.1 CMM

3.4.1.1 Lateralization of Fusiform Cells

Our data indicate that performing an auditory discrimination task is associated with increased lateralization of migrating new neurons in the CMM of male zebra finches. Though electrophysiological and IEG studies have reported changes in CMM because of

discrimination training, this is the first study to report an increase in the relative number of new, migrating neurons in this region as a result of exposure to an auditory discrimination task. This finding suggests that increased functional demands brought on by the discrimination task can increase lateralization of neuronal recruitment to CMM, which has similarly been suggested about the effect of operant discrimination on neurogenesis in NCM (Tsoi et al., 2014; Newman, 2022). Indeed, while previous research shows that both NCM and CMM are involved in long-term memory formation and storage and auditory discrimination, operant learning has differential effects on neural responses in the two regions: CMM maintains higher levels of neural responses to reinforcement-predictive stimuli compared to novel stimuli, while NCM responses habituate to familiar stimuli (Bell, Phan, & Vicario, 2014). If lateralization of recruitment of new neurons to NCM and CMM are indeed tied to functional demands, it could be that the relatively long timeframe of the current discrimination task and high volume of trials could present a greater asymmetry of functional demands in CMM than in NCM. In other words, recruitment of more new neurons to the right hemisphere of CMM supports ongoing cognitive processing during continued discrimination of reinforcement-predictive stimuli. Further, given that the acoustic stimuli used in this task are devoid of their social relevance, NCM may have played less of a role in discriminating these stimuli compared to the naturalistic stimuli used in previous studies implicating NCM in auditory discrimination.

3.4.1.2 Lateralization of Stain Coverage

Although there were no significant differences in the number of new migrating and differentiating neurons between the groups, we observed a higher degree of

lateralization in DCX-ir stain coverage in the CMM of birds in the yoked control group. This suggests that mere exposure to auditory stimuli can affect some aspects of neuronal maturation (e.g., dendritic arborization) without affecting the number of new neurons. It is difficult to discern whether lateralization of DCX-ir coverage in the present study can be attributed to mere repeated exposure to auditory stimuli; similar effects have been reported with visual stimuli in the visual cortex of rats, but to our knowledge, the relationship between mere exposure to acoustic stimuli and neuronal maturation has not been studied in songbirds (Ge et al., 2007). This finding highlights the importance of considering the effects of learning tasks on different stages of neurogenesis (e.g., migration, differentiation, and maturation), and similarly, which stages of neurogenesis may be affected by absence of naturalistic stimuli, like social interactions. Indeed, in the current study, the lack of lateralization of DCX-ir stain coverage in the control group could represent suppression of neuronal maturation below baseline, rather than increased functional demands caused by mere exposure in the yoked control group.

3.4.4 NCM

3.4.4.1 Lateralization of Fusiform Cells

In the discrimination group, we found that task performance was positively correlated with the number of DCX-ir fusiform cells in the right hemisphere of the ventral NCM. Further, we found that the relative difference in neuron number between the right and the left hemisphere was positively correlated with discrimination task performance. Tsoi et al. (2014) also showed that DCX-ir hemispheric asymmetry was associated with discrimination performance, but in their study, the authors found that having more new neurons in the left hemisphere than the right was positively correlated

with task performance, which indicates lateralization in the opposite direction of what we observed in the present study. However, our current finding is in line with previous research examining functional dissociations in the left and right hemispheres of NCM. Indeed, neurons in the left hemisphere of NCM are more selective to conspecific song than those in the right hemisphere, whereas neurons in the right hemisphere of NCM are more selective to non-vocal sounds than those in the left hemisphere (Theunissen et al., 2004; Phan et al., 2006; George et al, 2008). This could indicate that the stimuli used in the current study, which are devoid of their social relevance, are processed in the right hemisphere of NCM and require new neurons to store new memories; hence, birds with more new migrating neurons there have a performance advantage over birds with fewer new migrating neurons. It is worth noting, however, that some birds in the current study and Tsoi et al.'s (2014) study had hemispheric asymmetries favouring the opposite hemisphere than where the effect was observed, so this could indicate that the direction of lateralization depends on individual subjects' learning strategies. Indeed, several studies show that songbirds, including zebra finches, respond differently to rewarded and punished stimuli, with some birds showing a preference for rewarded stimuli and others showing preference for avoiding punished stimuli (Delius & Emmerton, 1990; Bolhuis et al., 2004; Chen et al., 2016). Indeed, neural asymmetries in auditory perceptual regions, whether considering cell count, type, or activation, are context-dependent and may undergo experience-dependent change throughout life. Future studies should attempt to control for birds' previous social contexts and life histories by using age-matched subjects from a breeding colony (i.e., using siblings in each group). This will allow for

assessment of individual variability in learning strategies and a more robust idea of hemispheric asymmetries in performance of auditory perceptual tasks.

3.4.6 Sex Differences

It is noteworthy that the influence of task performance on the lateralization of new migrating neurons was observed solely in male birds. While the suggested function of CMM in the storage of auditory memories appears to be applicable to both male and female songbirds, the behavioural significance of tasks may influence how neurogenesis in modulated. For example, male zebra finches can outperform females when discriminating between two different syllables (Gentner et al., 2001), but female zebra finches can outperform males when discriminating between conspecific calls (Van der Kant et al., 2021). However, although female zebra finches can outperform males on some discrimination tasks we did not observe sex differences in task performance, nor did we expect any based on findings from the study on which the task was based research (Kriengwatana et al., 2016; van Heineken et al., 2014), so it is unlikely that the sex difference observed in the present study relates to sex differences in task performance, rather it could be a difference in the basic dynamics of neurogenesis in male compared to female birds. The existing literature on sex differences in neurogenesis among songbirds is inconclusive: some studies find greater neurogenesis in males (Kirn et al., 1999; Heimovics et al., 2009; Boumans et al., 2020), others greater neurogenesis in females (Guigeno et al., 2016; Shevchouk et al., 2017), and still others suggesting no discernible sex differences (See Balthazart & Ball, 2021 for review). Additionally, research on sex differences in neurogenesis among songbirds usually concentrates on HVC and RA.

While the sex difference observed in CMM in the current study is difficult to reconcile with the current literature, it is worth noting that testosterone treatment can reverse the effects of social isolation in suppressed neurogenesis (Lipkind et al., 2002; Riters & Alger, 2004); therefore, a possible explanation of the current finding is that male birds had better preparedness to recover from, or were less affected by, the effects of social isolation than females. In other words, our findings suggest that the auditory discrimination task could mitigate the impact of social isolation on supressed lateralization of new migrating neurons in male birds, but not in females. Although sex differences in neurogenesis among songbirds are not fully understood, exploring the differences in birds' capability to recover from the effects of social isolation is a crucial area for future research, as it can broaden our understanding of the complex interplay between biological, psychological, and social factors that affect brain health.

3.4.7 Social Isolation

Numerous studies report that social isolation can reduce neurogenesis in adult songbirds including zebra finches (Lipkind et al., 2002; Riters & Alger, 2004; London & Clayton, 2008; Adar & Nottebohm, 2011), but there are some exceptions (Barnea & Nottebohm, 1994; Kirn et al., 2001; Vyssotski et al, 2002). In this study, we predicted that the control group would exhibit suppressed neurogenesis compared to the other groups, and that engaging in the discrimination task or simply hearing the stimuli would rescue this effect. However, we did not detect any between-group effects on our dependent measures. It could be that neither the discrimination task nor mere exposure to the stimuli were sufficient to mitigate effects of social isolation experienced by birds in all conditions (except where noted above). Indeed, studies investigating these effects

typically rely on manipulations that involve physical isolation from conspecifics and subsequent reintroduction to them in order to produce effects of social isolation, change, and enrichment. This raises the question of whether social isolation across groups in the current study impaired our ability to detect any enrichment effect that the discrimination task may have otherwise produced. In other words, social isolation may have suppressed neurogenesis across all groups, which may explain why we did not observe as robust changes in new neuron migration and differentiation as seen in birds with normal baseline levels of neurogenesis. For future studies investigating a task's impact on neurogenesis in a particular brain region, it might be beneficial to limit training to a few hours per day and return birds to a social environment between training sessions to ensure that birds in both the discrimination and yoked groups can maintain regular and normal levels of social interaction and preserve their baseline level of neurogenesis.

3.4.8 Future Directions

This study is the first to report that operant discrimination of auditory stimuli can enhance lateralization of new migrating neurons in an auditory perceptual region of adult songbirds. Findings from this study further implicate the role of adult neurogenesis in acquiring and maintaining auditory memories. Our study used a challenging operant discrimination task that several birds did not show improvement on through the course of the experiment. We chose this task specifically because it was difficult (van Heijningen et al., 2013), and tasks requiring long-term engagement are necessary to encompass all stages of neurogenesis. However, a possible by-product of this task was that it caused suppression of neurogenesis below baseline, which makes sense given the sensitivity of neurogenesis to social factors, especially in a highly social animal like the zebra finch. To

draw more precise inferences about a task's potential to upregulate neurogenesis or rescue the effects of isolation, task performance should be restricted to a few hours per day to maintain normal social context in the experimental and yoked groups. Future studies should also consider using other histological markers to determine whether new neurons produced by engaging in the task become functionally integrated to existing circuits.

3.4.9 Conclusion

Our study contributes to understanding the complex interplay between sex differences, social environment, and neurogenesis in songbirds. We found that social isolation had differential effects on the lateralization of neurogenesis in the NCM of male and female birds. Specifically, males showed a recovery in lateralization after an auditory discrimination task, while females did not, suggesting that lateralization of neurogenesis in NCM may be influenced by sex-specific factors and task demands. Our findings underscore the importance of considering sex differences and social context in studies investigating neurogenesis and lateralization in songbirds. Further investigation into the impact of different types of tasks, such as reward-based versus punishment-based learning, on lateralization of neurogenesis could provide valuable insights into the cognitive processes involved in discrimination tasks in songbirds. Understanding the factors that influence lateralization of neurogenesis in songbirds has implications for our understanding of brain plasticity, cognitive processes, and the role of social context in shaping neural development and function.

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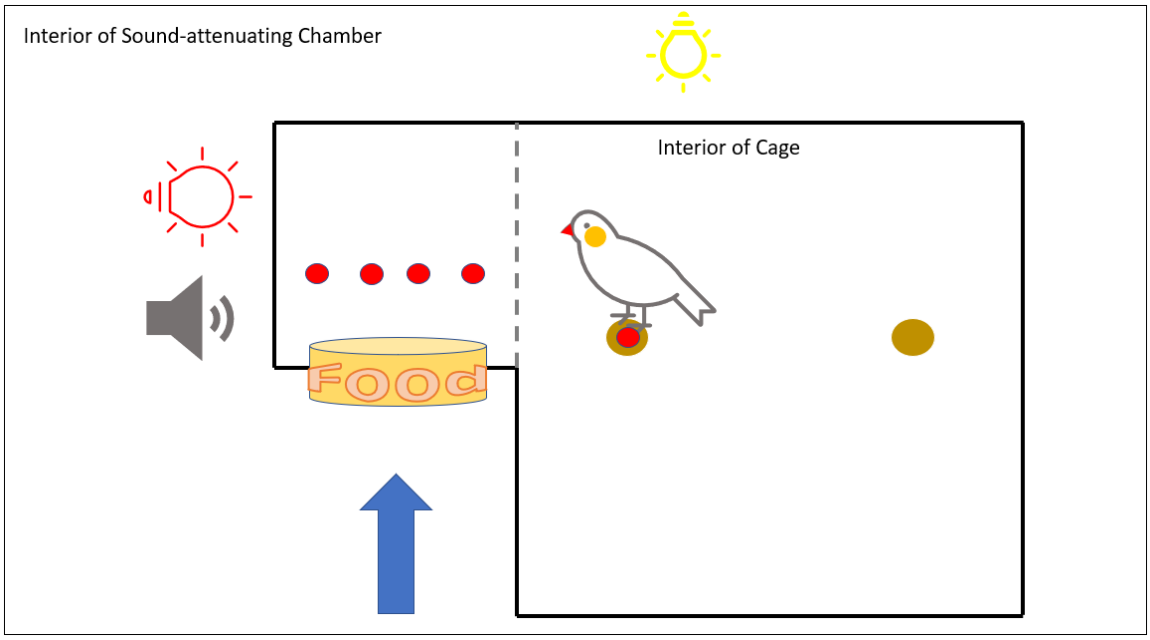

Figure 3.1. Side view of operant chamber. A standard bird cage was placed inside a sound-attenuating chamber next to a custom feeder. The perch and feeder area were equipped with infrared sensors (red circles) to monitor birds' location. When birds accessed the feeder after a rewarded stimulus, the motorized hopper ascended. When birds accessed the feeder after an unrewarded stimulus, the house light extinguished. A speaker adjacent to the feeder broadcast auditory stimuli, and a red light was mounted within the rear wall of the feeder.

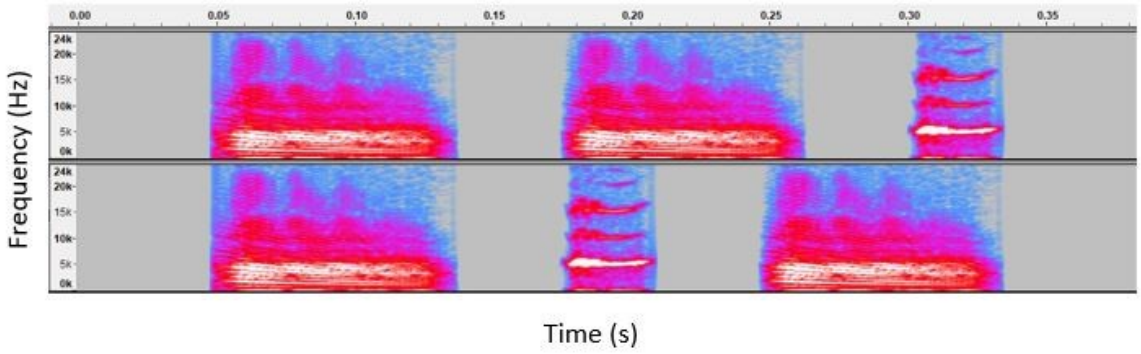

Figure 3.2. Sample spectrograph of auditory stimuli. Rewarded stimuli (bottom) contained non-repeated note patters (e.g., ABA), while punished stimuli contained a repeated note (e.g., AAB; top). Each birds in the experimental group had a stimulus set containing 6 stimuli made up of two song elements.

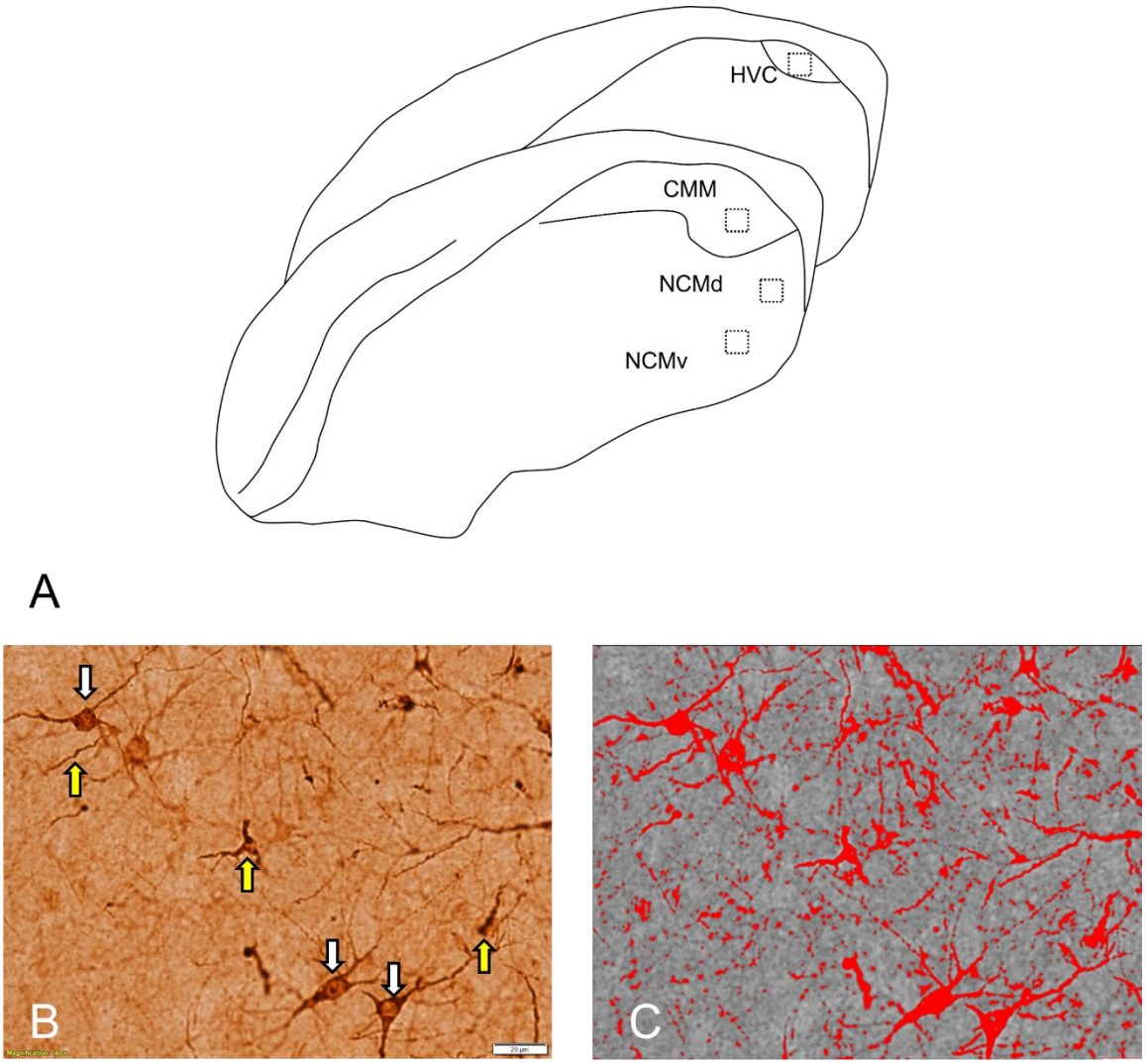

Figure 3.3. Schematic of sampling regions and sample images of DCX-ir zebra finch tissue. Panel A shows sampling regions for HVC, NCMd, NCMv, and CMM in sagittal sections of brain tissue. Panel B (left) shows fusiform (yellow arrows) and multipolar (white arrows) in a sample image from CMM. Panel C shows the same image with the percent of tissue immunoreactive for DCX staining colored red. The primary DCX antibody was run at 1:250 and images were captured at 40x magnification.

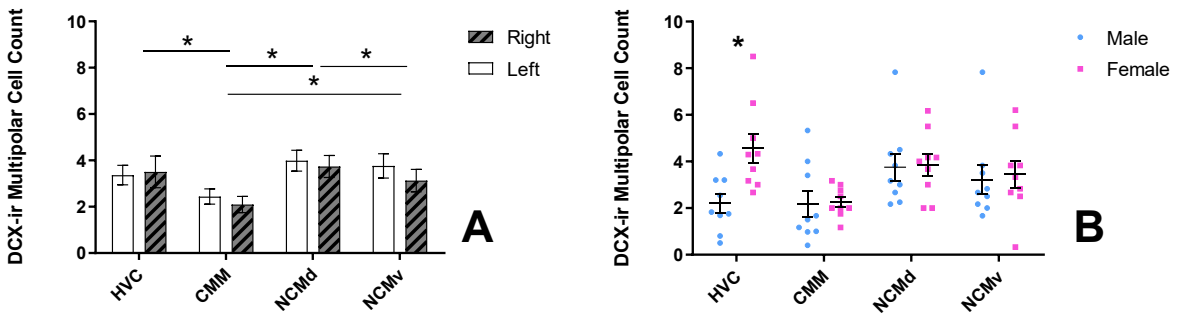

Figure 3.4. Regional and sex differences in the mean number of DCX-ir multipolar cells in zebra finch brain tissue. Panel A shows differences in the number if multipolar cells between brain regions. Panel B shows that female birds had more multipolar cells in HVC than male birds. Asterisks indicate significant effects at the $p<0.05$ level. Error bars represent standard error.

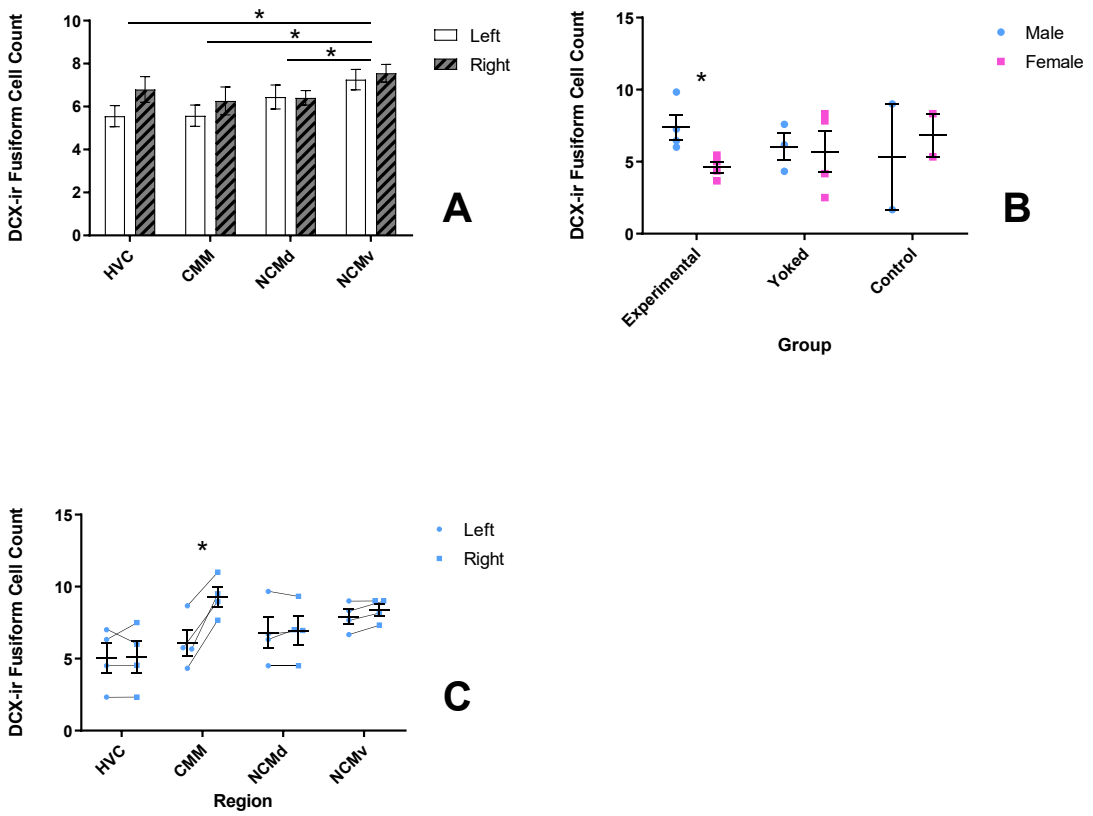

Figure 3.5. Regional, group, and sex differences in the mean number of DCX-ir fusiform cells in zebra finch brain tissue. Panel A shows regional differences in the number of fusiform cells. Panel B shows that males in the experimental group had more fusiform cells in CMM than females. Panel C shows that male birds had more fusiform cells in the right hemisphere of CMM than in the left. Asterisks indicate significant effects at the $p<0.05$ level. Error bars represent standard error.

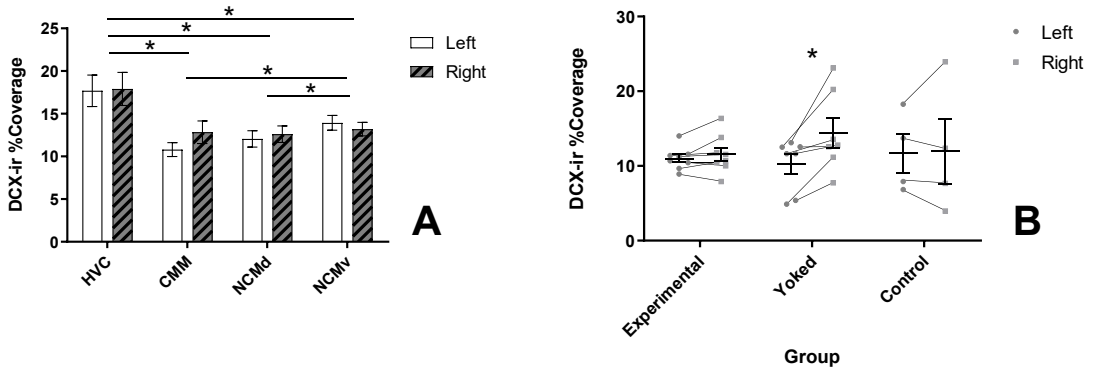

Figure 3.6. Regional and group differences in the DCX-ir staining coverage of images of zebra finch brain tissue as a percentage. Panel A shows regional differences in the average stain coverage. Panel B shows that birds in the yoked group had greater staining coverage in the right hemisphere of CMM than in the left.

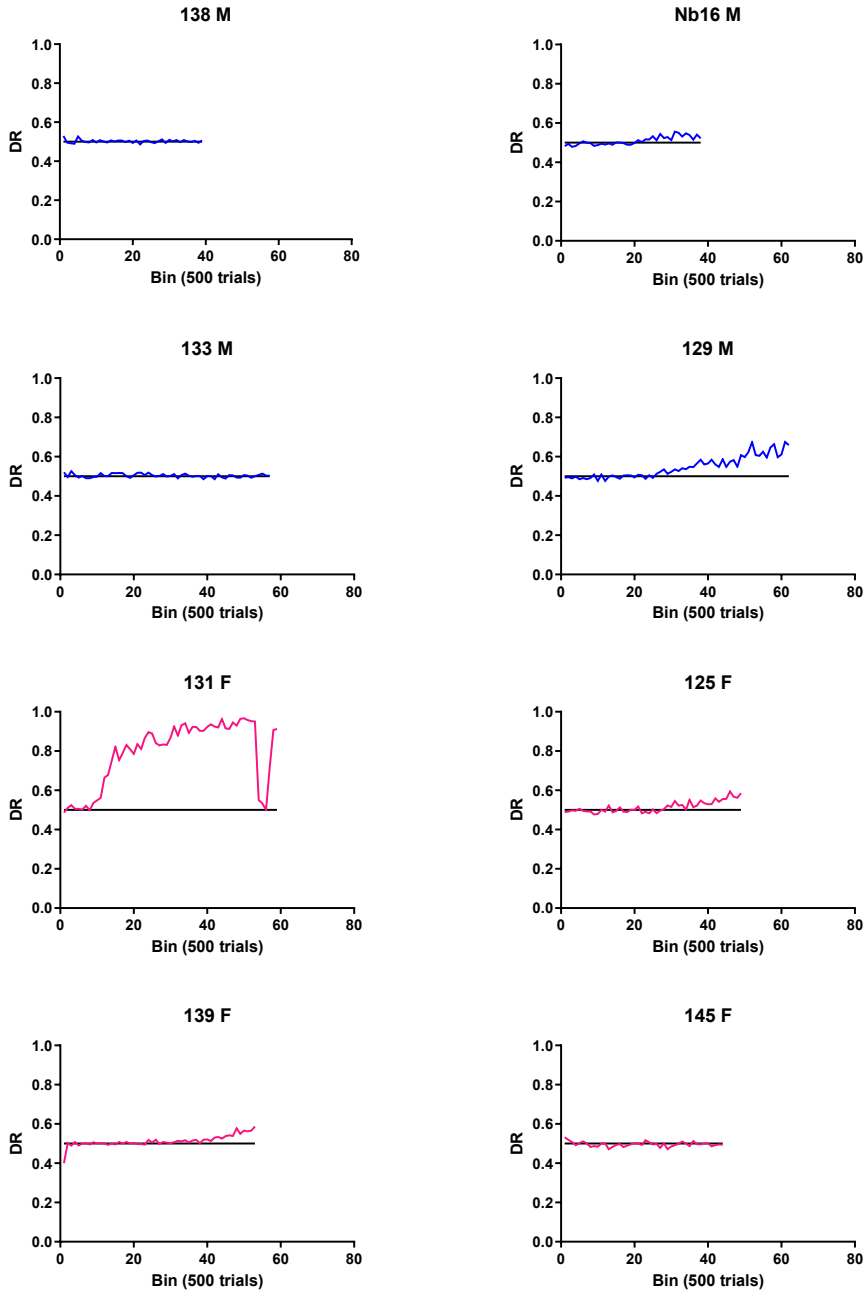

Figure 3.7. Discrimination performance of zebra finches in the experimental group. The discrimination ratio (DR) was calculated in bins of 500 trails. Performance across bins is indicated using a blue line for males and a pink line for females.

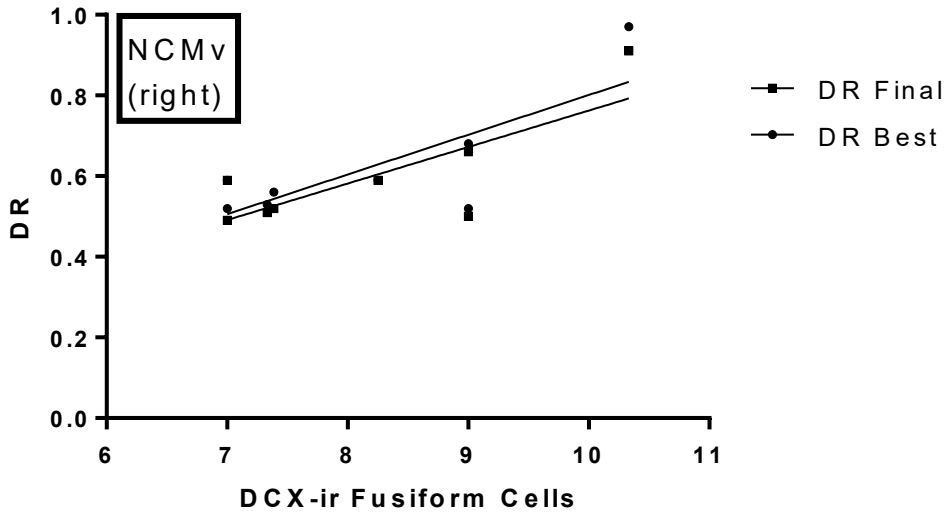

Figure 3.8. Pearson correlation between the number of fusiform cells in the right hemisphere of the ventral region of NCM and discrimination performance of zebra finches. Birds' best (r=0.73) and final (r=0.75) discrimination ratios were strongly correlated with the number of fusiform cells at the p<0.05 level.

CHAPTER 4: SEASONALITY OF THE EUROPEAN STARLING (STURNUS VULGARIS): EFFECTS OF SEASON ON STRESS AND NEUROGENESIS IN AUDITORY PERCEPTUAL BRAIN REGIONS

4.1 Introduction

Songbirds adapt their behaviour to seasonal changes in their natural environment (Sharp, 1984). This flexibility allows birds to time their reproductive behaviour for when environmental conditions are conducive to offspring vitality (Dawson, 2008). A primary example of seasonally variable behaviour is vocalizing; a predominantly male behaviour, birds vocalize most frequently during spring when they are defending territory and courting mates (Krebs et al., 1978; Eens et al., 1991). During fall, songbirds of many non-migratory species vocalize less frequently and shift their behavioural strategy toward flocking, to defend against predators, forage for food, and conserve energy (Davis, 1970). These shifts in behaviour, and parallel shifts in birds' endocrine status, are predictably regulated by photoperiod. In spring, increasing day length coincides with an increase in gonadotropin-releasing hormone (GnRH), leading to gonadal maturation; after breeding, a decrease in GnRH accompanies rapid gonadal regression (Farner & Follett, 1966; Wingfield & Farner, 1978; Dawson et al., 2001). Overall, the ability of songbirds to adjust their behaviour in response to seasonal changes highlights the remarkable plasticity of their nervous system and endocrine regulation and underscores the importance of studying their neurobiology in a naturalistic context.

Seasonal changes in the environment are also associated with changes in birds' stress response. Plasma corticosterone (CORT)—the primary stress hormone in birds—increases in response to environmental stressors like inclement weather, which may function to inhibit reproductive behaviour until favourable conditions resume (Wingfield

et al., 1994; Wingfield et al., 1995; Astheimer et al., 1995). However, much of the research showing a relationship between stress and breeding condition comes from arctic species, who have a small window of opportunity for breeding and thus may rely more heavily on acute fluctuations in plasma CORT to time breeding than birds from temperate climates, who spawn multiple clutches in a breeding season. In temperate European starlings (*Sturnus vulgaris*), for example, CORT remains high throughout winter and breeding season, with a decrease in summer before increasing again in fall (Dawson & Howe, 1983; Romero & Remage-Healey, 2000). Nonetheless, there are two major challenges in measuring CORT levels: the diurnal fluctuations in plasma CORT and the rapid increase of CORT upon capture, making it difficult to obtain baseline measurements (Romero & Reed, 2005). An alternative measure of stress and immune function in birds is the heterophil:lymphocyte ratio (HLR) (Huber et al., 2021). Unlike CORT, HLR returns to baseline levels within 30 minutes after exposure to handling stress. If HLR shows a strong correlation with CORT measures, it could serve as a viable alternative or complementary measure when studying the relationships between behaviour, stress, and neurogenesis.

In addition to seasonal changes in behaviour and physiology, songbirds experience seasonal changes in their brain, particularly in the vocal control system—a series of brain nuclei responsible for vocal learning, perception, and production (Brenowitz et al.,1997). HVC is a vocal system nucleus involved in all three processes, and predictably, it undergoes seasonal plasticity along with behaviour and physiology. One measure of plasticity is neurogenesis—the proliferation, differentiation, migration, and incorporation of new neurons to neural circuits. Studies show that levels of

neurogenesis in HVC fluctuate across season, but there are differences between species and between sexes. In subtropical, non-migratory Indian weaverbirds (*Ploceus philippinus*) and temperate, migratory redheaded buntings (*Emberiza bruniceps*), doublecortin (DCX) immunoreactivity (ir)—a measure of new neuron migration, differentiation, and maturation—in HVC of male birds is highest when they are in breeding condition, with some differences between species in terms of the types of new cells observed (Surbhi et al., 2016). This suggests that neurogenesis in HVC may be linked to birds' annual reproductive strategy (i.e., increased vocalizing), regardless of other aspects of their life histories (i.e., seasonal migration). However, in temperate, non-migratory red-winged blackbirds (*Agelaius phoeniceus*) and brown-headed cowbirds (*Molothrus ater*), males have more DCX-ir in HVC during non-breeding than in breeding condition, suggesting that neurogenesis in HVC in these species supports song addition of new vocal elements after breeding (Guigueno et al., 2016). Female cowbirds had the opposite pattern of seasonal change to males: DCX-ir was higher in HVC during breeding season, which may suggest that neurogenesis underlies different processes in male and female birds.

Two auditory regions, caudomedial mesopallium (CMM) and caudomedial nidopallium (NCM) are involved in auditory processing and memory formation (Pinaud & Terleph, 2008; Bell et al., 2014). In male birds, who learn their song from a tutor, the strength of neural activation in NCM is positively correlated with the degree of similarity of their own song to their tutor's (Bolhuis et al., 2000). Further, the accuracy of song and strength of auditory memory is associated with neurogenesis in NCM; that is, male birds with more accurate copies of their tutor's song and stronger memories for recently heard

songs have a higher degree of hemispheric asymmetry of new neurons than birds with poorer copies and weaker memories (Tsoi et al., 2014). In females, CMM is responsible for storing the memory of their father's song; however, the degree of neural activation in NCM is positively correlated with the length and complexity of male's songs, suggesting that NCM plays a role in females' preference for males with longer and more complex songs (Terpstra et al., 2006; Mountjoy & Lemon, 1996; Gentner et al., 2001; Gentner & Hulse, 2000).

Despite accruing evidence for the involvement of these regions in performance of seasonally variable, social behaviour, few studies have looked at seasonal changes in their structure or function. Surbhi et al. (2016) compared seasonal neuroplasticity in two species with different annual life history states (LHSs): the non-migratory weaverbird (*Ploceus philippinus*) and the migratory redheaded bunting (*Emberiza bruniceps*). They found that weaverbirds had the highest number of DCX-ir fusiform cells (new, migrating neurons) in NCM in pre-breeding condition, but no seasonal differences were detected for CMM. Buntings, on the other hand, had the highest number of DCX-ir round cells (new, differentiating neurons) in NCM during breeding season and the highest number of DCX-ir round cells in CMM during pre-breeding and breeding season. For DCX-ir fusiform cells, counts were highest in pre-breeding and breeding condition in NCM but did not differ in CMM. Their results suggest that there is some species difference in seasonal neurogenesis in auditory perceptual regions; however, the potential behavioural correlates of these difference are not discussed, and this phenomenon has yet to be studied in a non-migratory species where male birds add to their song repertoire throughout the lifespan.

The primary objective of this study was to examine seasonal variations in neurogenesis in HVC, CMM, and NCM of male and female European starlings. The study aimed to test three hypotheses: the activation hypothesis, which suggests that increased neurogenesis supports the performance of seasonal reproductive behaviours such as singing and discrimination; the learning/stereotypy hypothesis, which proposes that neurogenesis aids in the consolidation of new song elements after breeding; and the maintenance hypothesis, which suggests that neurogenesis is involved in the acquisition and retention of auditory memories throughout life. Based on the activation hypothesis, we expected higher levels of neurogenesis in the HVC of male birds during the breeding season, as they engage in courtship, territory defense, and exhibit more complex and frequent singing. For female birds captured in breeding season, we expected to observe increased neurogenesis in the CMM and NCM, which would underlie increased demands required for discrimination between potential partners. Under the learning/stereotypy hypothesis, we predicted a relative increase in HVC neurogenesis in male birds during the non-breeding season when they add new song elements to their repertoire, while females were expected to exhibit stable neurogenesis in HVC across seasons. Finally, under the maintenance hypothesis, we anticipated seasonally stable rates of neurogenesis in the CMM and NCM of both male and female birds.

A secondary purpose of this study is to assess the relationship between stress and neurogenesis and the relationship between two measures of stress: plasma CORT and heterophil:lymphocyte ratio (HLR). Here, we test two alternative hypotheses: First, stress is associated with increased neurogenesis, and second, that stress impedes neurogenesis. Given that our blood samples were collected within the time range identified by Romero

and Reed (2005; i.e., within 3 min of capture), we expect our two stress measures to be positively correlated.

4.2 Method

4.2.1 Subjects

Sixteen male (*n*=10) and female (*n=6)* European starlings (*Sturnus vulgaris*) were captured in either May (*n*=8, 6 males) or December (4 males) 2018 on campus at Dalhousie University in Halifax, Nova Scotia. We captured birds using potter traps baited with various food items, including Doritos, Hickory Sticks, and French fries. All procedures were approved by the University Committee on Laboratory Animals (UCLA; protocol #17-120) and birds were captured under a permit from Nova Scotia Department of Natural Resources (Wildlife division).

4.2.2 Blood Collection and Preparation

When a bird entered the potter trap, a timer was started. Birds were retrieved immediately for blood collection. Using a 26G needle, a small puncture was made in the brachial vein, and blood was collected into a maximum of six 100 µl microcapillary tubes; we stopped collecting blood at 3 min after the timer was started. The tubes were sealed with Critoseal and stored in a cooler filled with ice until transferred to the lab. After bleeding, eight hackle feathers were removed to determine birds' age, and birds were placed in cloth bags and transferred to the lab within 1 h. At the lab, we prepared blood smears by placing a drop of whole blood from one capillary tube onto a microscope slide and using another slide to produce a smear. After drying, the slides were fixed and stained using the Hema 3 procedure (Fisher) and coverslipped.

The remaining capillary tubes were centrifuged for 10 min, and the proportion of red blood cells to plasma was measured using a hematocrit reader. Plasma was extracted from the tubes and stored at –80C until processing.

4.2.3 Blood

We analyzed blood smears using an Olympus CX23 microscope with oil immersion at 1000X magnification. We identified the monolayer of the blood smear and worked from the top of the slide down, marking the bottom of each field of view (FOV). When the bottom of the FOV was reached, we started a new one, and when we reached the bottom of the smear, we moved laterally toward the end of the smear. For each FOV, we counted the white blood cells (WBC) and identified them each as either basophil, eosinophil, heterophil, lymphocyte, or monocyte. We estimated the number of red blood cells (RBC) by counting the number of RBC on one half of the FOV, switching between sides for each FOV. We repeated this process until we counted 100 WBCs. From these data, we estimated the number of WBCs per 10,000 RBCs, and we calculated the heterophil:lymphocyte ratio (HLR).

4.2.4 Corticosterone assay

We used a commercially available enzyme-linked immunosorbent assay (ELISA) kit (Enzo Life Sciences; cat no. ADI-900-097) to quantify total corticosterone (CORT) in plasma. This ELISA kit has been used extensively to quantify plasma CORT in songbirds (e.g., Deviche et al., 2014). All plasma samples were diluted to 1:40 according to the small-volume protocol described by the manufacturer; briefly, 10 μL of steroid displacement reagent (diluted 1:100 in distilled water) was added to 10 μL of raw plasma and then diluted with 380 μL of assay buffer. All samples were assayed in duplicate; all

samples with a coefficient of variation greater than 20% (n=2) were excluded from analyses. One sample did not run and was excluded from analyses. An additional sample was excluded from analysis due to being more than two standard deviations outside of the mean and inconsistent with previously published data (Romero & Remage-Healy, 2000). Plasma samples from two birds (both non-breeding) read below the minimum sensitivity of the assay (prior to adjusting for the dilution factor); these data were adjusted to the minimum sensitivity (27 pg/mL; 1080 pg/mL adjusting to dilution factor) in our statistical analyses. We did not assess inter-assay variation as all samples were assayed on one plate; intra-assay variation is reported by the manufacturer as 8.0%.

4.2.5 Age

We measured hackle and iridescence length to the nearest 0.1 mm using a dissecting microscope. Hackle length was measured from the hackle tip to the base of the main shaft of the feather, and iridescence was measured from the tip to where no iridescence was present, following previous research (Kessel, 1951). Birds were assigned to either second year (SY) or after second year (ASY) depending on iridescent length, according to the method established by Kessel (1951). Males were considered SY if their iridescence length was < 11.0 mm, and ASY it was > 11.0 mm. Females were considered SY if their iridescence length was < 6.5 mm, and ASY if it was > 6.5 mm.

4.2.6 Perfusion

Upon transfer to our facilities, birds were weighed and then killed via lethal injection of 1:1 Xylazine:Euthanyl. Birds were transcardially perfused with heparinized PBS followed by 4% paraformaldehyde. Brains were harvested and placed in paraformaldehyde for two days, followed by one day in 30% sucrose in PBS. The

following day, brains were frozen on pulverized dry ice and stored at -80C until sectioning. Gonadectomies were performed to identify birds' sex and breeding condition. Testes were measured using a Vernier caliper and ovaries were examined qualitatively to determine their stage.

4.2.7 Immunohistochemistry

Brains were sectioned in the coronal plane using a cryostat (-17°C; 12° blade angle) at a thickness of 50 μm into cryoprotectant (composition) and stored at –20C until immunohistochemistry was performed. Before processing, sections were removed from the freezer and washed five times in PBS to remove cryoprotectant solution. All washes were 5 min with agitation, and all steps were carried out at room temperature unless stated otherwise. Tissue was first incubated in H_2O_2 for 30 min. It was then washed three times in PBS and incubated in 10% normal horse serum in 0.3% PBS with Triton (PBS/T) for 1 h. DCX (C-18) primary antibody (Santa Cruz, sc-8066) was prepared in 0.3% PBS/T at a concentration of 1:250; tissue was incubated overnight at 4˚C. The following day, tissue was washed twice in .01% PBS/T and then incubated in biotinylated horse anti-goat secondary antibody (Vector, BA-9500) at 1:400 in 0.3% PBS/T for 1 h. The tissue was then washed three times in .01% PBS/T before being incubated in avidin-biotin horseradish-peroxidase (Vector, ABC Elite) at a concentration of 1:200 in 0.3% PBS/T for 1 h. Again, tissue was washed twice in 0.1% PBS/T. The antibody complex was visualized using SIGMAFAST DAB (Sigma-Aldrich) for 90 s, then washed five times in PBS. Tissue was float mounted onto gelatin-coated slides and coverslipped with Permount. We used negative controls (primary antibody omitted) and positive controls (tissue known to be positive for primary antibody) to determine non-specific binding.

4.2.8 Microscopy and Quantification

We captured images at 40x magnification using an Olympus BX-51 microscope and CellSens software. Three images were captured per hemisphere from each of caudomedial mesopallium (CMM) and caudomedial nidopallium (NCM), and HVC. Figure 1 shows the sampling area for each region of interest. In each region, we quantified three types of cells: multipolar (Mp), round (r), and fusiform (f). Multipolar cells exhibited distinct cell bodies with multiple processes emanating from them, while round cells lacked observable processes (Hall & MacDougall-Shackleton, 2012; Taufique et al., 2018). Between the two, multipolar cells are thought to be at a later stage of maturation (Balthazart & Ball, 2014). Fusiform cells had elongated cell bodies with spindle-shaped processes, which is indicative of neuronal migration. Refer to Figure 2 for a representative image where each cell type is identified. Each cell type was counted exhaustively within each image and averaged across images for each region. Further, we used the 'threshold' function in ImageJ to quantify the area of the image that was DCX-ir (%Cov). Measuring DCX-ir %Cov (Figure 3C) allowed us to estimate morphological characteristics not accounted for by cell counts, like cell size and extend of dendritic arborization. Therefore, we used DCX-ir %Cov as a proxy of neuronal maturation.

4.2.9 Statistical analyses

We ran a season x sex x region x hemisphere ANOVA for each of our dependent measures: DCX-ir fusiform cell count, DCX-ir multipolar cell count, DCX-ir round cell count, and DCX-ir % Coverage. Further, we conducted correlational analyses to assess relationships between H:L and plasma CORT and our DCX-ir measures. Tests yielding a p value of 0.05 or less are considered statistically significant. Tests yielding a p value

between 0.05-0.1 are considered statistical trends. Partial eta squared (*ηp2*) is reported for significant ANOVAs for the purpose of comparisons among future studies of similar design.

4.3 Results

4.3.1 Physiology

We performed a t-test to determine whether testes volume of male starlings differed between breeding conditions. Males captured in spring had larger testes than those captured in fall, $t(8)=2.78$, $p=0.039$ (Figure 1). Of the two females captured in spring, one had a large primary follicle, while the other had several relatively large follicles. All female birds captured in fall had small, primordial follicles.

We also performed t-tests to assess differences in baseline plasma CORT levels and H:L ratio in whole blood across seasons. The tests revealed that birds captured in spring had higher plasma CORT concentrations (ng/mL) than those captured in fall, $t(11)=2.218$, $p=0.024$ (Figure 2A), but there was no difference in HLR between breeding and non-breeding birds (Figure 2B). No sex differences were detected for these measures. We performed correlation analyses to determine whether CORT and H:L were related, but no correlation was detected, $r=0.020$, $p=0.950$ (Figure 3).

4.3.2 DCX measures

Figure 4 shows the mean number of DCX-immunoreactive (ir) multipolar (panel A), fusiform (B), and round (C) cells and the mean DCX-ir percent coverage (%Cov) (D), separated by region and hemisphere and averaged across sex, while Figure 5 shows the same data separated by region, separately for Spring (Panel A) and Fall (Panel B). We

ran a region x hemisphere x season x sex ANOVA for each dependent variable. For tests that violated the assumption of sphericity, a Greenhouse-Geisser correction was applied.

For multipolar cells (Panel A), there were no main effects, but there was a significant region x sex interaction and a significant region x sex x season interaction; $F(2,16)=3.994$, $p=0.039$, $\eta p2=0.333$ and $F(2,16)=5.424$, $p=0.039$, $\eta p2=0.333$, respectively. Follow-up t-tests revealed that females had more DCX-ir multipolar cells in HVC than males; t(12)=-2.209, p=0.047, $\eta p2=0.583$ (Figure 5). We performed separate ANOVAs for male and female birds, which revealed that male birds had more DCX-ir multipolar cells in fall that in spring; $F(1,6)=12.681$ $p=0.012$, $\eta p2=0.679$ (Figure 6), but there was no difference in females. There were no other effects of season for male or female birds.

For DCX-ir fusiform cells (panel B), there was a significant main effect of region, $F(2,10)=29.65$, $p<0.001$, $\eta p2=0.748$. Simple main effects showed that there were more DCX-ir fusiform cells in NCM than in HVC ($p<0.001$) and CMM ($p<0.001$) but there was no difference in number of fusiform cells between HVC and CMM. There were no other main effects. There was a significant region x season interaction, $F(2,10)=7.345$, $p=0.004$, $\eta p2=0.423$. We conducted t-tests to compare differences in cell counts between regions for birds in each season separately. Birds captured in both spring and fall had more DCX-ir fusiform cells in NCM than in CMM; $t=-5.712$, $p=0.001$ and $t=-11.240$, $p<0.001$), respectively. However, only birds captured in spring had more DCX-ir fusiform cells in NCM than in HVC ($t=-0.9.449$, $p<0.001$; Figure 6A), and only birds captured in fall had more DCX-ir fusiform cells in HVC than in CMM ($t=2.588$, $p=0.049$; Figure 6B). There were no other interactions for fusiform cells.

For DCX-ir %Cov (Figure 7, panel C), there was a main effect of region, $F(2,20)=14.480$, $p<0.001$). Simple main effects showed that there was greater DCX-ir %Cov in NCM than in HVC ($p=0.006$) and CMM ($p<0.001$). There were no other main effects or interactions.

We ran correlation analyses among all subjects to determine whether our stress measures were associated with neurogenesis. We found strong, positive correlations between plasma CORT concentration (ng/mL) and the number of DCX-ir multipolar cells in CMM ($r=0.840$, $p=0.001$; Figure 7A) and between plasma CORT concentration (ng/mL) and the number of DCX-ir round cells in the right hemisphere of CMM ($r=0.799$, $p=0.006$; Figure 7B). We also found a moderate, positive trend between plasma CORT concentration (ng/mL) and the number of DCX-ir round cells in NCM ($r=0.590$, $p=0.073$; Figure 7C).

4.4 Discussion

The purpose of this study was to assess seasonal changes in neurogenesis in the auditory perceptual regions of the temperate, non-migratory European starling. A secondary purpose was to assess the relationship between stress and neurogenesis in these regions and whether our two stress measures are related. Overall, birds captured in spring exhibited higher plasma CORT concentrations than those captured in fall, suggesting that CORT supports biological and behavioural processes required in breeding season. Correlation analyses revealed significant relationships between plasma CORT levels and the number of DCX-ir round cells in specific brain regions, suggesting a potential link between stress and neurogenesis. Furthermore, DCX measures demonstrated more nuanced effects, with sex-specific differences observed in the number of multipolar cells and region-specific differences in the abundance of fusiform cells. These findings provide

valuable insights into the physiological and cellular mechanisms underlying behavioural processes in songbirds and highlight the interplay between hormonal factors and neurogenesis in the context of behaviour.

4.4.1 Season, CORT, and H:L

We found that birds had higher levels of plasma CORT in spring than in fall, which is consistent with the previous findings in starlings and other free-living species (Dawson & Howe, 1983; Wingfield, 1994). Indeed, during spring, birds are more likely to be engaged in reproductive behaviour including courtship, mating, nest building, and raising offspring, all of which have been shown to correlate with CORT in some songbird species. In starlings, Love et al. (2004) found that female starlings who abandon their nest have higher baseline plasma CORT than those who do not; therefore, it could be that the performance of seasonally relevant reproductive behaviours drive effects on CORT levels, rather than more broad constructs like season or breeding condition (Dawson & Howe, 1983; Romero & Remage-Healey, 2000). In the current study, the variability in our CORT measures in birds captured in spring may be indicative of their engagement or disengagement in an array of seasonally relevant reproductive behaviour. Future studies should assume a more ecologically sound approach to investigating relationships between season and hormone levels; for example, by observing the nesting status and territoriality of individual subjects.

In contrast to plasma CORT levels, H:L did not show a predictable change across seasons in European starlings. This suggests that a consistent seasonal variation in H:L ratio may not be expected in this species. The H:L ratio reflects not only stress but also immune function and is influenced by various hormonal and environmental factors. To

gain a more comprehensive understanding of the specific environmental factors that influence the H:L ratio, it would, again, be beneficial to incorporate a more detailed observation of the birds' natural behaviour prior to blood and tissue collection. While it may not exhibit predictable seasonal changes, it provides a holistic perspective on the interplay between stress, immune response, and other physiological factors. Therefore, when studying the stress response and its relationship to environmental factors, it is essential to consider both plasma CORT levels and the H:L ratio to gain a more comprehensive understanding. However, it is important to note that while H:L ratios provide insights into stress, they cannot be considered a substitute for measuring plasma CORT levels in capturing predictable seasonal effects on stress.

4.4.2 DCX

4.4.3 HVC

In HVC, males exhibited higher levels of neurogenesis during spring compared to fall. This increase was specifically observed in multipolar cells, although a similar non-statistically significant trend was also noticed in fusiform cells. During fall, the number of DCX-labeled fusiform cells in HVC was greater than in CMM, but this difference was not observed in the spring. Instead, during the spring season, there were more DCX-labeled fusiform cells in NCM than in HVC. These findings are consistent with Guigueno et al.'s (2016) study, where the authors found that male red-winged blackbirds and brown-headed cowbirds had more HVC neurogenesis in non-breeding than in breeding condition. Our results offer further support for the *learning/stereotypy* hypothesis, suggesting that increased neurogenesis in male starlings in non-breeding condition supports the addition of new song elements to their repertoires, and a decrease in

neurogenesis during breeding condition permits increased song frequency and stereotypy (Kirn & Nottebohm, 1993; Kirn et al., 1994; Tramontin & Brenowitz, 1999).

4.4.4 Sex Differences

In contrast to Guigueno et al.'s (2016) findings, we did not observe a seasonal pattern of neurogenesis in female birds that was opposite to that of males; instead, we found no significant seasonal difference. However, we did find that female birds had a higher number of DCX-labeled multipolar cells in HVC compared to males. This finding is consistent with Hall and MacDougall-Shackleton's (2012) finding in European starlings, though unlike their study, we did not find a significant effect of sex for fusiform cells in addition to multipolar ones. Hall and MacDougall-Shackleton (2012) proposed that the high rates of neurogenesis in females may be related to song perception, particularly the recognition of male conspecifics (Gentner & Hulse, 2000). Previous studies have shown that songbirds exhibit better discrimination performance during the summer, suggesting a seasonal change in their ability to memorize conspecific vocalizations (Cynx & Nottebohm, 1992). And given that neurogenesis has been associated with improved auditory discrimination performance, a seasonal variation in neurogenesis may be expected to coincide with the seasonal change in behaviour (Tsoi et al., 2014). However, the absence of seasonal differences in neurogenesis observed in our study suggests that the increased neurogenesis in female birds in HVC may be related to behavioural flexibility required to meet changing demands throughout the year, rather than a single functional demand such as song learning in males. Female starlings have highly variable songs (Pavlova et al., 2005), sing throughout the year, discriminate between male conspecific songs during breeding season (Mountjoy & Lemon, 1996), and

form pairs with female conspecifics in winter, all of which involve activation of HVC (Hausberger et al., 1995; Henry et al., 2013). Our study, conducted with free-living subjects, is more likely to capture the variability of demands in naturalistic environments compared to laboratory studies. However, by not measuring the birds' behaviour, our ability to speculate about the behavioural correlates of neurobiological effects is limited. Laboratory and controlled field experiments will be useful in determining whether increased neurogenesis in female birds compared to males provides them with the readiness to shift between these behaviours throughout the year.

4.4.5 NCM and CMM

We found that the relative level of neurogenesis between perceptual regions, NCM and CMM, was consistent across season; that is, birds had significantly more DCX-ir fusiform cells and DCX-ir %Cov in NCM than in CMM in both spring and fall. This finding supports the *maintenance* hypothesis, where CMM and NCM require consistent levels of neurogenesis to form and maintain memories necessary for social interactions throughout birds' life histories. Meaning, despite CMM- and NCM-dependent behaviours varying with season, the behaviours rely on similar neurobiological substrates. Reproductive behaviours during spring and flocking and pairing behaviour during fall constitute a suite of social behaviours that may require sustained neurogenesis in the perceptual regions, so despite the behavioural change, the demands on the neurobiological substrates are consistent. This is in line with previous findings that neurogenesis in CMM and NCM are associated with social behaviour, which starlings perform in both breeding and non-breeding condition. Our findings, however, are not consistent with Surhbi et al. (2016), who found that neurogenesis in these regions varied

by season, though no explanation of those findings was offered. In their study, the authors sampled birds at four discrete life histories, which likely provided more precise estimates of neurogenesis at those points than our study did. Further research on variation in the rate of neurogenesis in the perceptual regions of free-living birds in warranted to determine whether observed changed are due to changes in social behaviour.

4.4.6 CORT and DCX

We found that high levels of plasma CORT were associated with an increased number of DCX-ir round cells in the left hemispheres of NCM and HVC. In our study, we identified round cells as cells that, unlike MP cells, do not have characteristic processes. In Hall et al. (2014), however, these cells are counted exclusively in hippocampus (Hp). It is possible that our identification of round cells in HVC could be conflated with our counts of MP cells, as some images may lack the level of focus required to identify processes; however, in NCM, these cells appeared frequently in our sample's tissue without the defining characteristics of MP cells, and they appear morphologically similar to the round Hp cells identified in Hall et al. (2014). It has been reported that corticosterone inhibits neurogenesis (Newman et al., 2010); however, data around this effect is conflicting (Wada et al., 2014), and a clear picture of this relationship in free-living birds has not been established. It could be the case that stress differentially affects migrating and differentiating neurons, but the characteristic differences between cell types in different brain regions has not been established. Future studies should examine the effects of CORT on specific cell types through the different stages of neurogenesis.

Previous research has shown hemispheric asymmetry in the rates of neurogenesis in NCM; specifically, birds that are better learners have a greater degree of hemispheric

asymmetry of DCX-labeled neurons (Tsoi et al., 2014). Further, in starlings, the degree of hemispheric asymmetry of HVC activation is positively associated with social integration; that is, birds with a greater degree of lateralization of neuronal response in HVC to conspecific song have more social partners than birds with weaker lateralization of neuronal responses (Cousillas et al., 2020). Again, it would be interesting to further investigate this effect in relation to behaviour; it is not unlikely that multifactor interactions between behavioural (e.g., nesting), environmental (e.g., weather), and endocrinological (e.g., CORT) factors covary with changes in the brain.

4.4.7 Conclusion

This study is the first to describe naturally occurring changes in DCX expression in auditory perceptual regions of the European starling. Further, this is the first study to report a correlation between plasma CORT and DCX-ir in HVC and NCM of free-living birds. One shortcoming of our study is our small sample size. In spring, we observed a high degree of variability in our dependent measures, which could reflect variability in the nesting status of birds. To overcome this shortcoming, future studies examining the effects of breeding on stress and neurogenesis should integrate behavioural measures.

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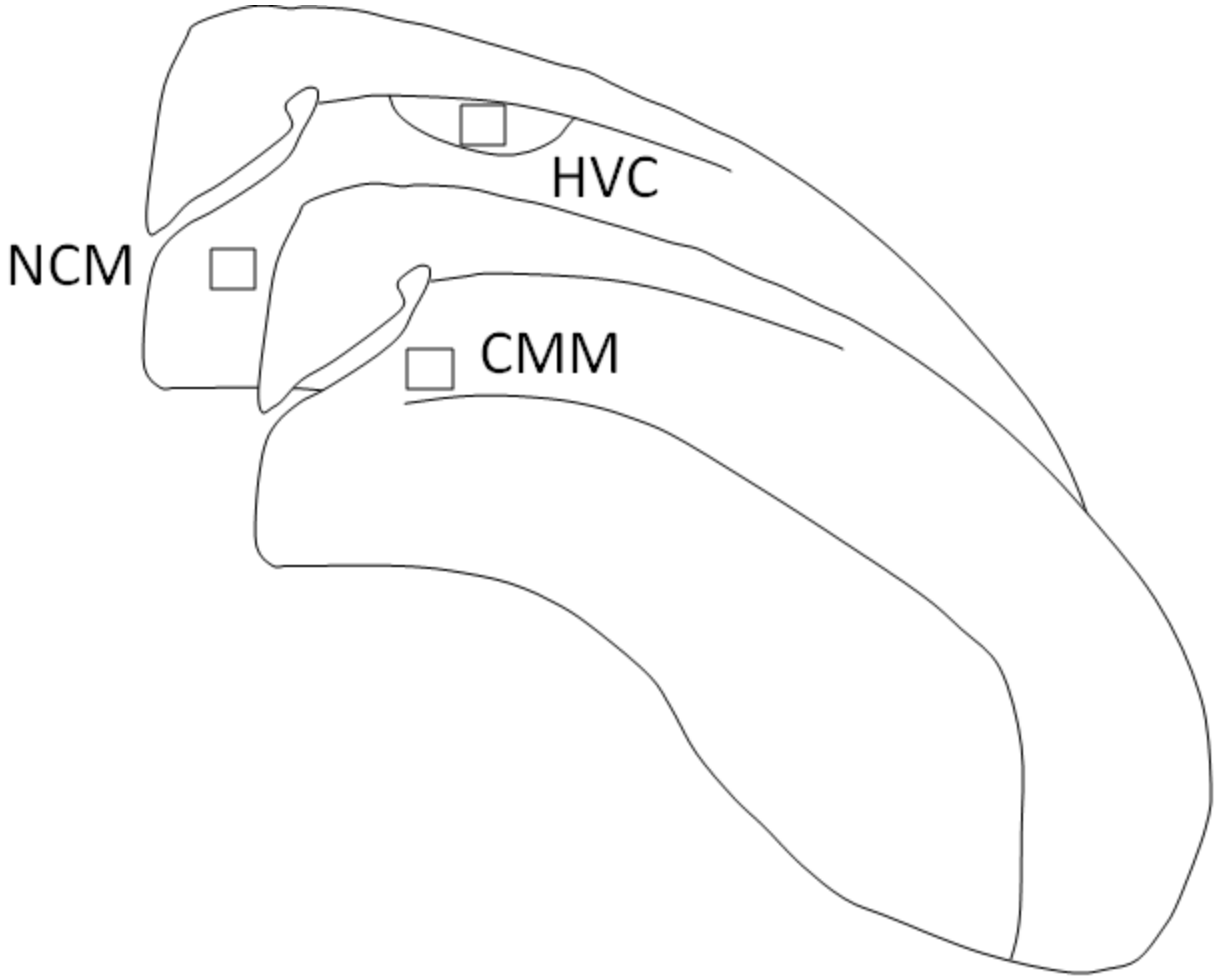

Figure 4.1. Coronal sections of European starling brain with sampling regions. Images were taken at 40x magnification.

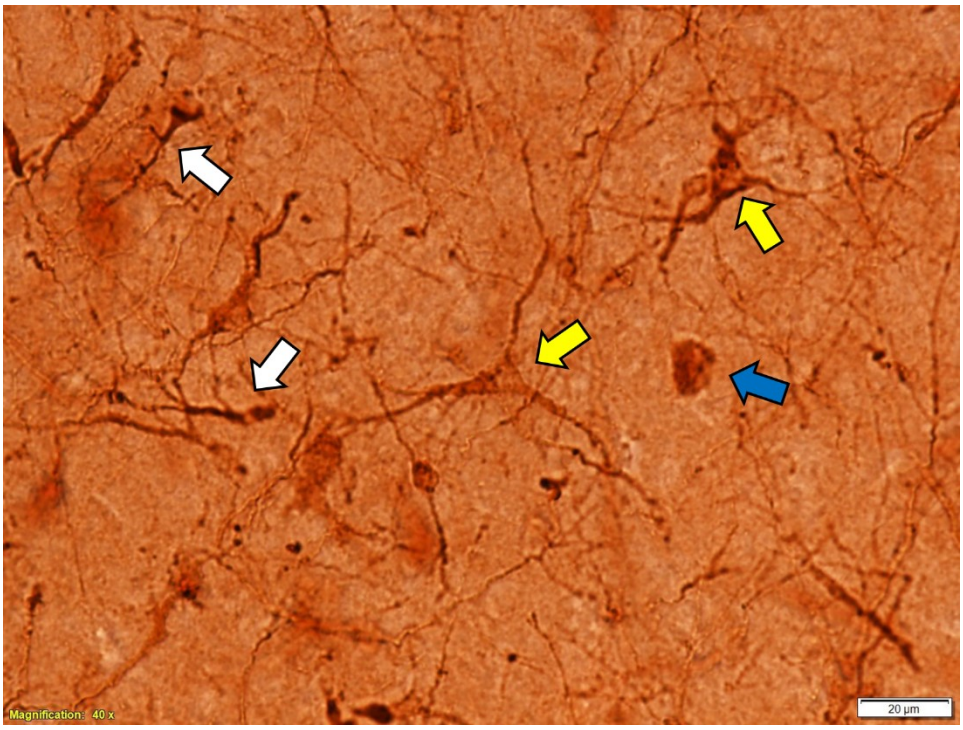

Figure 4.2. Representative image of DCX-immunoreactive tissue in taken from the NCM of a European starling at 40x magnification. Primary DCX antibody was run at a concentration of 1:250.White, yellow, and blue arrows indicate the presence of fusiform, multipolar, and round cells, respectively.

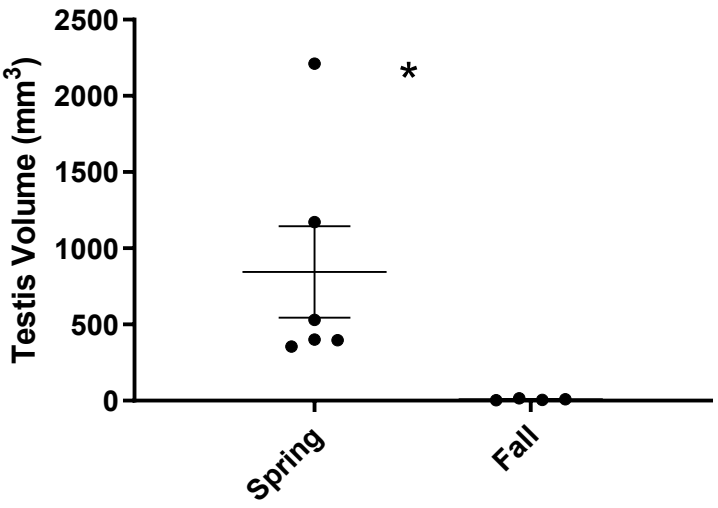

Figure 4.3. European starlings captured in spring had larger testes than those captured in fall, p=0.039. Horizontal lines represent group means and error bars represent standard errors.

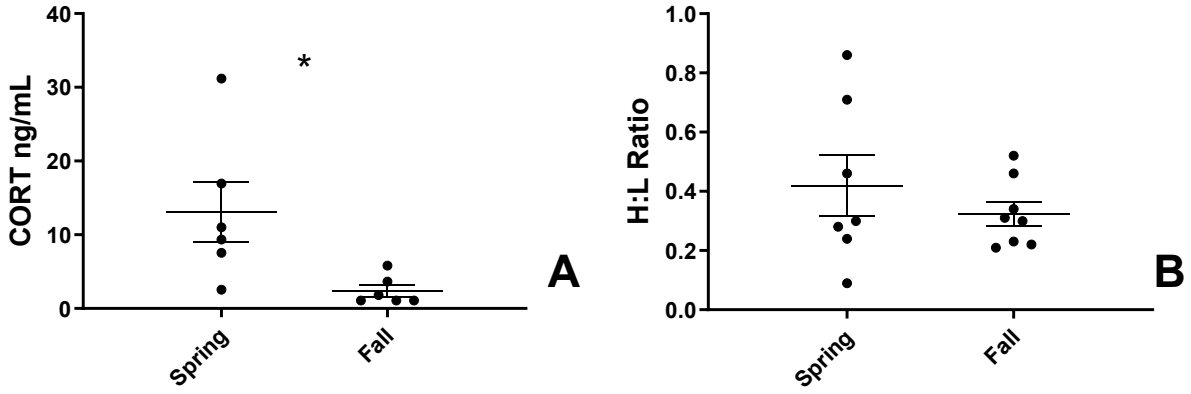

Figure 4.4. Panel A shows baseline plasma corticosterone levels in individual male and female starlings captured in spring and fall. Birds captured in spring had greater plasma CORT concentration than those captured in fall. Panel B shows the H:L ratio in whole blood (number of heterophils to lymphocytes) for male and female starlings captured in spring and fall. No seasonal difference in H:L ratio was detected. Horizontal lines represent group means and error bars represent standard errors. Asterisk indicates a significant effect at the $p<0.05$ level.

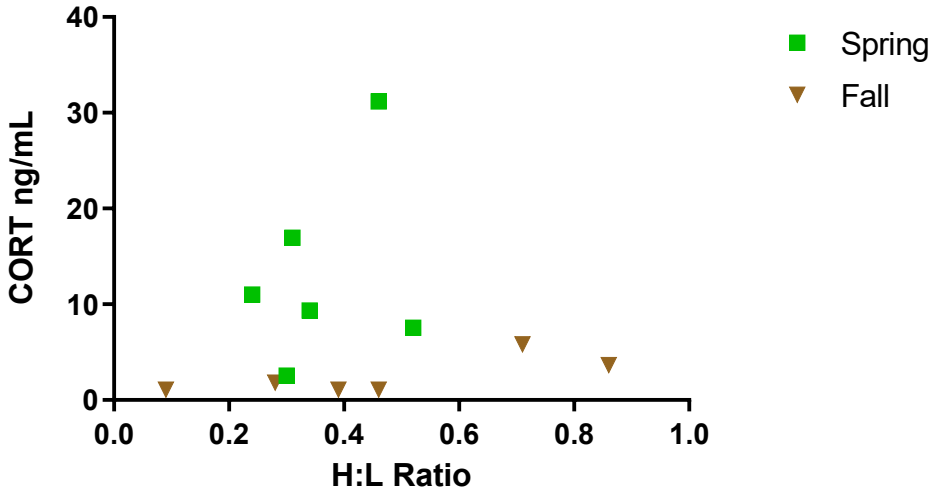

Figure 4.5. There was a small, non-significant Pearson correlation between plasma corticosterone (CORT) and heterophil:lymphocyte ratio (H:L) ratio in European starlings, r=0.205, p=0.501.

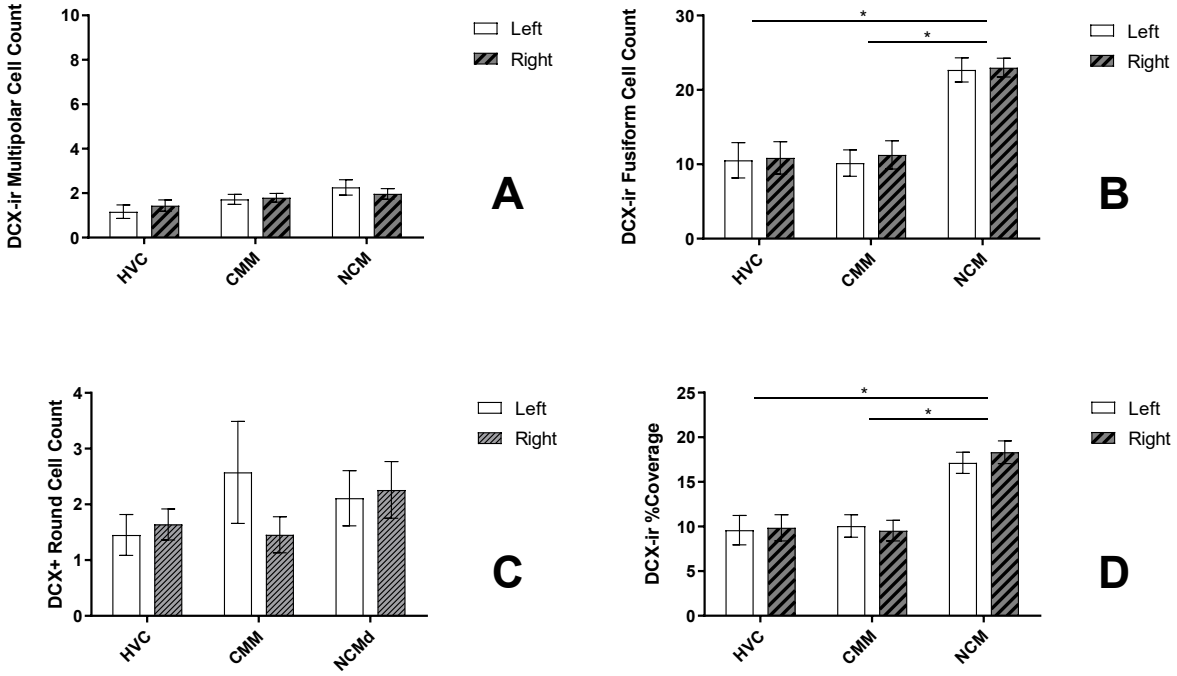

Figure 4.6. Panel A shows that European starlings had more DCX-immunoreactive (ir) fusiform cells in NCM than in CMM and HVC. Panel D shows that birds had greater staining coverage (DCX-ir %Cov) in images taken from NCM than from CMM and HVC. Bars represent means and error bars represent standard errors. Asterisks denote significant effects at the $p<0.01$ level.

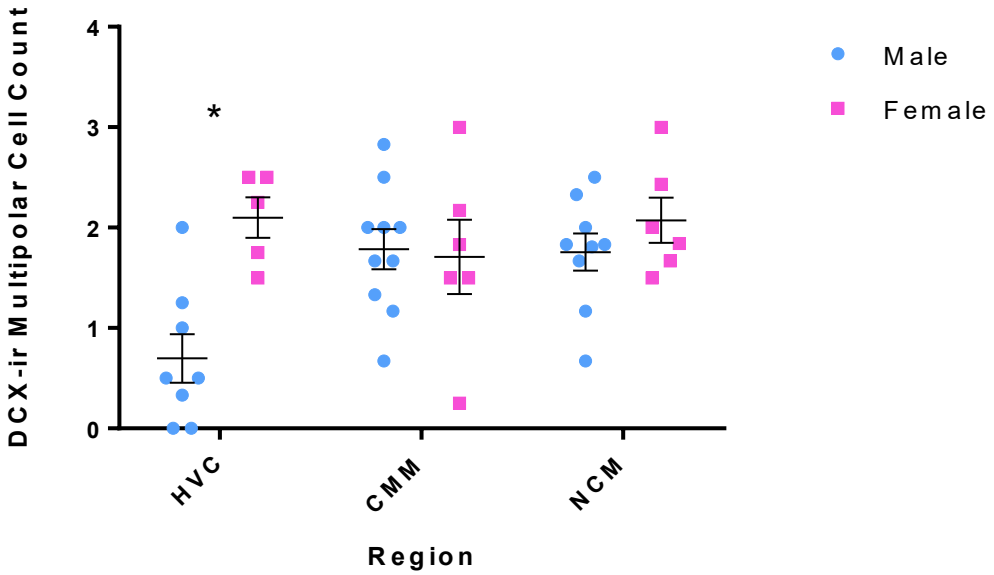

Figure 4.7. Male European starlings (blue circles) had fewer DCX-immunoreactive (ir) multipolar cells in HVC than females (pink circles). Cells were counted exhaustively in each image and averaged over three images per region, per hemisphere. Horizontal lines represent group means and error bars represent standard errors. Asterisk denotes a significant effect at the $p<0.05$ level.

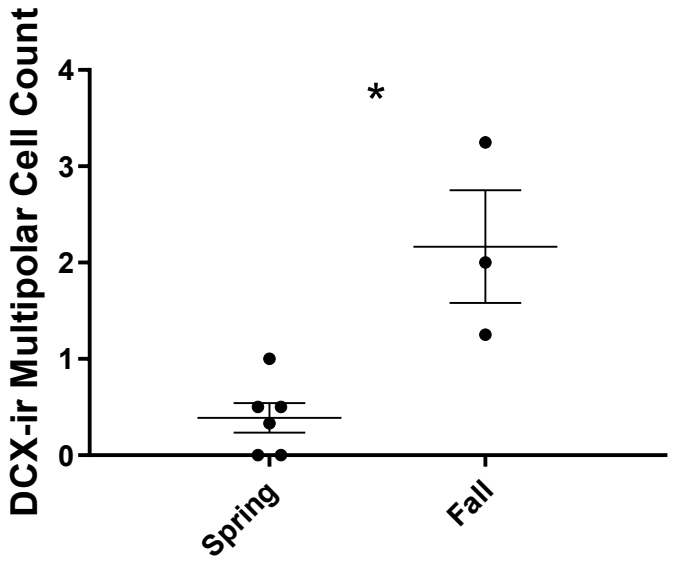

Figure 4.8. Male European starlings had more DCX-immunoreactive (ir) multipolar cells in HVC during fall than in spring. Cells were counted exhaustively in each image and averaged over three images per region, per hemisphere. Horizontal bars represent group means and error bars represent standard errors. Asterisk indicates a significant effect at the $p<0.05$ level.

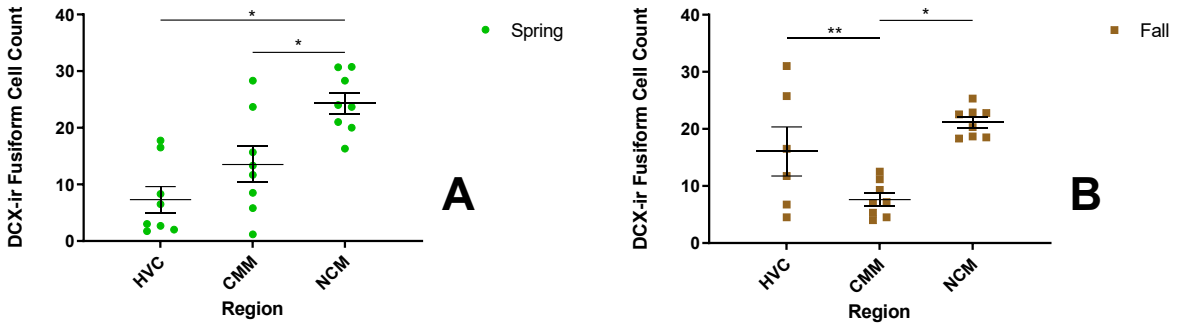

Figure 4.9. Panel A shows that European starlings captured in spring had more DCX-immunoreactive (ir) fusiform cells in NCM than in CMM and HVC, whereas panel B shows that birds in fall had more DCX-ir fusiform cells in NCM and HVC than in CMM. Cells were counted exhaustively in each image and averaged over three images per region, per hemisphere Asterisks denote significant effects at the $p<0.001$ (*) and $p<0.05$ (**) levels.

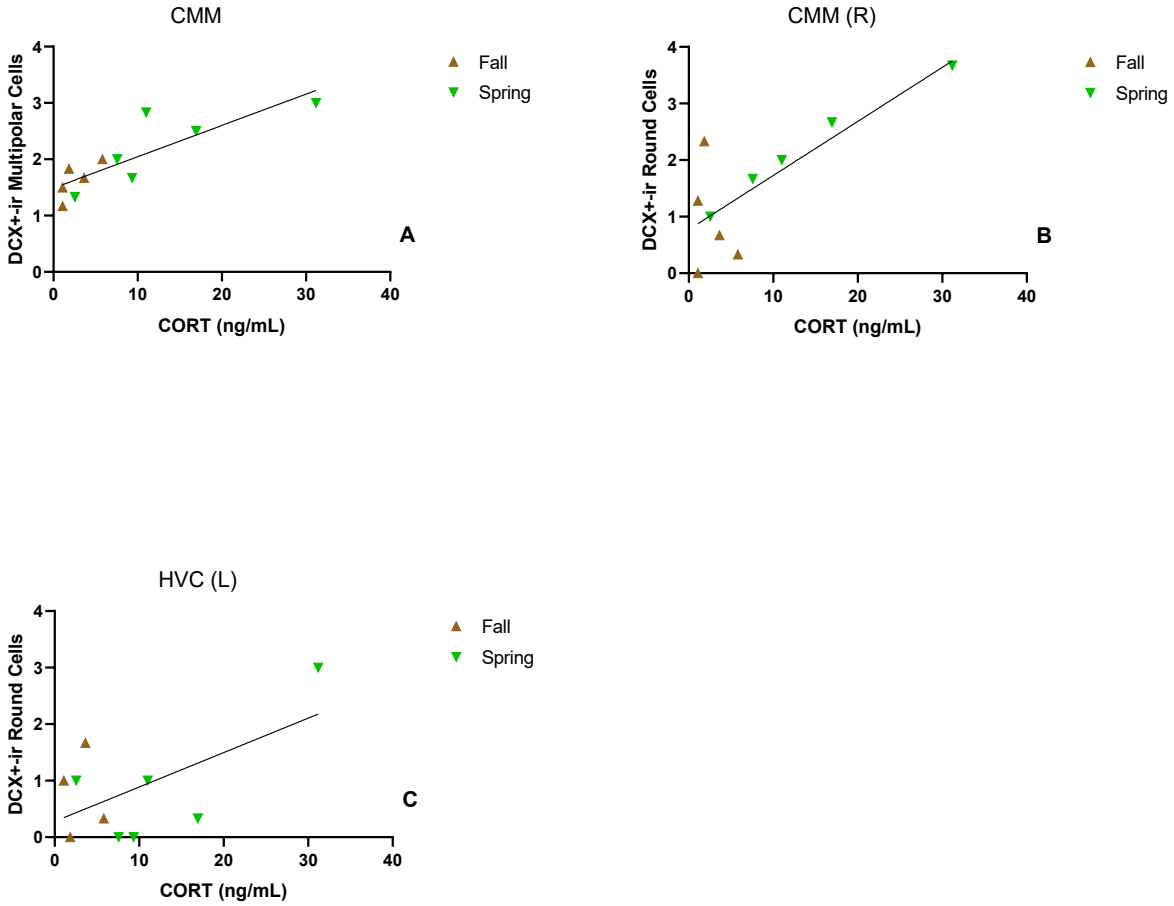

Figure 4.10. Corticosterone (CORT) concentration in European starlings was strongly correlated with the number of DCX-ir round cells in the left hemispheres (LH) of HVC (A) and moderately correlated with the number of DCX-ir round cell in the LH of NCM (B), r=0.779; p=0.005 and r=0.591; p=0.043, respectively. Panel C shows a moderate, non-significant correlation between H:L and the number of DCX-ir round cells in NCM (averaged between hemispheres), r=0.442; p=0.087.

Chapter 5: General Discussion

The first aim of this research was to visualize and quantify regional differences in DNA methylation in the songbird brain by adapting a 5-mC labeling protocol used in rodents. We performed immunohistochemistry to label 5-mC in the brains of three songbird species: black-capped chickadees, European starlings, and zebra finches. Although the staining quality was not as optimal as reported in the seminal study, we found that in chickadees, cells in the caudomedial mesopallium (CMM) exhibited greater 5-mC cell-stain intensity compared to cells in the caudomedial nidopallium (NCM). This difference in 5-mC intensity may contribute to the distinct roles of CMM and NCM in auditory memory and discrimination. No significant regional differences in 5-mC levels were detected in zebra finches or European starlings at the cell level, but some subtle differences in 5-mC levels at the image level were observed in finches. I provided recommendations for improving the immunohistochemistry protocol and made suggestions for future research.

In the second study, I investigated the impact of social isolation on neurogenesis in vocal and auditory brain regions of songbirds and asked whether engaging in an auditory discrimination task would modulate those effects. I used a note pattern task and an endogenous marker of neurogenesis to analyze effects on the presence of neuronal subtypes in HVC, CMM, and NCM. Contrary to our hypotheses, we found no significant increase in the migration, differentiation, or maturation of new neurons among birds performing the task, or listening to another bird perform the same task, compared to birds in complete isolation. However, there were some more nuanced impacts on neurogenesis within groups: male birds who performed the task had more DCX-ir fusiform cells in the

right than the left hemisphere of CMM. We also found that passive exposure to auditory stimuli was associated with more DCX-ir staining coverage in the right than in the left hemisphere of CMM. Moreover, the presence of more DCX-ir fusiform cells in the right hemisphere of NCMv was linked with improved discrimination performance, which aligns with previous research. These findings have the potential to inform future research in on the role of neurogenesis in discrimination of different types of auditory stimuli and the environmental factors that influence it.

In the third study, I captured male and female starlings in the wild during breeding (spring) and non-breeding (fall) seasons to compare rates of intermediate stages of neurogenesis. To minimize confounding effects related to captivity, birds were not held for more than one hour before being sacrificed. Our findings support the hypothesis that increased neurogenesis in HVC of male birds during the fall facilitates the addition of new song elements to their repertoire. In contrast, we propose that the lack of seasonal change in neurogenesis in auditory regions reflects the role of neurogenesis in conspecific discrimination throughout the year. We also suggest that higher neurogenesis rates in HVC of female birds, compared to males, may contribute to their behavioural flexibility. Notably, we report a relationship between stress and neurogenesis in CMM for the first time. These findings lay the foundation for investigating the specific link between neurogenesis and season-dependent and independent behavioural adaptations in songbirds.

5.1 Study 1: Measuring Global DNA Methylation In Auditory Brain Regions Of Three Songbird Species

DNA methylation involves the addition of a methyl group to cytosine nucleobases by DNA methyltransferases (DNMTs). The brain exhibits unique DNA methylation patterns compared to other tissues due to the diverse cell fates and gene expression programs required in neurons. However, the relationship between neurogenesis and regional or global DNA methylation patterns remains unclear due to limited neurogenic regions in the adult mammalian brain. The first study in this thesis explored the possibility of using histological, global DNA methylation as a measure of epigenetic modification in avian brain tissue. Despite the growing interest in the role of epigenetic processes in modulating learning and memory, songbird models have been explored to a lesser extent than mammalian ones (Shang & Bieszczad, 2022). Nonetheless, songbird models remain superior for investigating behavioural and neural plasticity, presenting ample opportunities for studying the role of epigenetic processes in learning and memory. The application of 5-mC immunohistochemistry is useful for precisely localizing methylation and co-labelling specific cell types. This technique is particularly promising for songbird models, where specific forms of learning and memory have been linked to dynamic, region-specific, neurobiological change; for example, spatial learning and memory in the hippocampus, song learning in HVC, and auditory discrimination in NCM and CMM. However, the epigenetic mechanisms that drive these changes remains unclear.

We detected a key regional difference in the staining intensity of 5-mC in cells of black-capped chickadees; specifically, we found that cells in CMM have more intense

staining than cells in NCM. This finding is compelling because of the physical proximity of these regions, which share a cytoarchitectonic border, and their related but distinct functions: Both CMM and NCM are involved in auditory discrimination, but where CMM is tuned specifically to learned sounds, NCM's function is less dependent on previous experience. This suggests that cells in these regions have distinct global methylation patterns, which may give rise to their dissociable functions. Indeed, in mammals, differences in patterns of DNA methylation are reflected in cell type and function; for example, in the rodent hippocampus, global DNA methylation is highest in the DG and CA1 regions and lowest in the CA2 and CA3 regions, which each of these regions being comprised of different cell types, receiving inputs from different regions, and ultimately serving a different role in learning and memory (Brown et al., 2008). Specifically, the CA1 is involved in memory consolidation, while the CA3 region is important for forming the context in contextual memory. To draw a parallel with the current findings, it could be that the higher levels of methylation observed in CMM reflect its role in consolidating memories of new, learned sounds, while the relatively lower level of methylation in NCM could reflect more contextual aspects of learning and memory, like identification of conspecifics in breeding and non-breeding seasons. This fits with findings from the mammalian literature where DNA methylation has been implicated in memory consolidation (Day & Sweatt, 2010; Zhao et al., 2010; Monsey et al., 2011). Specifically, this finding lends further support to the idea that regional differences in DNA methylation are a common feature of brain regions with dissociable functions and that these patterns are associated with different cell types and roles in learning and memory.

5.1.1 Limitations

The finding of different methylation levels between NCM and CMM, however, was not replicated in our zebra finch and European starling experiments. Given that the zebra finches were housed in the same operant training apparatuses as the black-capped chickadee, a certain level of environmental control would be expected; however, as we noted in Study 2, the finches may have experienced baseline effects of social isolation that affected DNA methylation—an effect that has been described in mammals (Siuda et al., 2014). Unlike the finches, the black-capped chickadees were returned to a single-housed caged in a room with other birds for several days before they were sacrificed, so any effects of social isolation may have been at least partially reversed after completing the experiment. Further, the black-capped chickadees all heard altered, natural stimuli, which may have lessened the effects of social isolation. It is possible that methylation patterns were more variable in black-capped chickadees because they were in a less isolated environment than the zebra finches.

5.1.2 Future Directions

Along with the methodological changes proposed in Study 1, future research should seek to establish regional differences in global DNA methylation of wild-caught birds or birds housed with adequate environmental enrichment. Unfortunately, the limited labeling quality observed in starling tissue precluded us from deriving reliable results from the tissue, but future research should also consider the potential effects of seasonal change on global DNA methylation.

5.2 Study 2: Operant Discrimination Of Acoustic Stimuli Differentially Affects Lateralization Of Doublecortin (DCX) Expression In Auditory Brain Regions Of Adult Male And Female Zebra Finches

Despite the lack of regional differences in DNA methylation observed between NCM and CMM in zebra finches, the research described in this thesis further supports the idea that neurogenesis contributes to auditory discrimination (Pytte, 2016; Tsoi et al., 2014). Social isolation can have profound effects on the brain, including suppressed neurogenesis (Alward et al., 2014). Indeed, we found that male birds exposed to an auditory discrimination task had increased lateralization of DCX-labeled fusiform cells in CMM, and that task performance was positively associated with increased lateralization of the same type of cells in NCM. Although the relationship between neurogenesis and auditory discrimination in NCM has been previously described, this is the first study to describe a similar (i.e., lateralized) effect in CMM. Since CMM responds more readily to a wider range of auditory stimuli than NCM, it could be that the discrimination required for this task placed greater functional demands on CMM, whereas the relationship between task performance and lateralization in NCM may be attributable to birds' performance at a phenotypic level. In other words, increased lateralization of neurogenesis in NCM could be a stable trait, whereas lateralization in CMM could be brought about by task performance. These findings paired with the regional differences in DNA methylation observed in chickadees suggest that CMM could play a more significant role in the consolidation of newly learned auditory stimuli, while NCM may be more sensitive to environmental and epigenetic effects during early development (i.e., song learning).

Using DCX, we were able to identify several distinct cell morphologies that are indicative of different, intermediate stages of neurogenesis. All cell types are thought to represent neurons after proliferation but before integration into their targeted neural networks. The two major cell types we focused on were fusiform and multipolar cells, with fusiform cells representing new, migrating neurons, and multipolar cells representing differentiating neurons (Balthazart & Ball, 2014). We also measured round cells in Chapter 4, which are thought to be in earlier stages of differentiation and are more readily observed in the hippocampus than in other regions. With regard to multipolar cells, we found that female finches and starlings had a greater number of cells in HVC than males, which fits with previous findings that female birds have greater neurogenesis in HVC compared to males. It has been argued that increased neurogenesis in female birds is associated with greater behavioural flexibility (Hall et al., 2014; Perkes et al., 2019). Indeed, we observed that the number of multipolar cells in HVC was greater in fall than in spring (Figure 4.9), suggesting that the dynamic nature of neurogenesis in this region could support the behavioural flexibility required for adaptive response to environmental change. However, given that we observed the relative difference in multipolar cells in vastly different experimental contexts, this would appear to be a stable trait across female songbirds.

With regard to fusiform cells, we found that both finches and starlings had a greater number of cells in NCM than in CMM, again, pointing to a difference in the neurobiological substrates of these functionally dissociable regions. This finding may suggest that fusiform cells underlie the processing of contextual sensory information, and thus are more abundant in NCM than in CMM. However, in Chapter 3, we found that

male birds in the experimental group had more fusiform cells in CMM than females and that fusiform cells were lateralized in this group. This targeting of fusiform cells in the CMM of male birds may point to increased demands on the neural substrates required for the formation and consolidation of new memories in male birds. In starlings, no such differences in fusiform cells were observed, which may lend further support to the idea that these effects are experimentally derived and not readily observed in free-living birds. Although this is the first study to report an association between discrimination and lateralization of new neurons in CMM, it does fit with the idea of lateralization being associated with discrimination performance, which we, and other studies, observed in NCM. In this case, however, lateralization of new neurons in CMM is not associated with performance, suggesting that mere exposure to the task was sufficient to increase the recruitment of new neurons, but not to facilitate learning. Overall, these findings suggest that different cell types play different roles in the neural substrates of memory formation, contextual processing, and behavioural flexibility. And while naturally occurring factors can be explained by species' life histories, some laboratory findings are not as easily resolved (Surbhi et al., 2016). Nonetheless, our experimental findings are foundational to further exploration of the differential roles of NCM and CMM in learning and perception.

5.2.1 Future Directions

The findings here warrant further investigation of the respective roles of NCM and CMM in discrimination learning and point to a specific direction for further research; specifically, investigating the differential effects of natural (i.e., song) compared to unnatural (i.e., pure tones without song-like characteristics) stimuli on neurogenesis in these regions. This is of particular interest considering the role isolation may play in

regulating neurogenesis in the types of operant paradigms described in this research. Another specific direction for future research would be to look more closely at aspects of neuroplasticity not accounted for by cell counts. Indeed, we found that DCX stain coverage was lateralized in the NCM of the yoked group, independent of lateralized cell counts. Measuring morphological aspects of DCX stained cells, such as cell size and the length and width of dendrites, could lend further insight into which neuroplasticity aspects are affected by exposure to and discrimination of auditory stimuli, and whether these aspects are differentially affected by isolation. Further investigating of sex differences in birds' ability to recover from the impacts of social isolation will improve our understanding of the intricate interconnections among psychological, social, and biological factors that influence brain health.

5.3: Study 3: Seasonality of the European starling (Sturnus vulgaris): Effects of season on stress and neurogenesis in auditory perceptual brain regions

Songbirds, such as the European starling (Sturnus vulgaris), exhibit complex vocal behaviour that changes across seasons. In spring, increasing day length triggers reproductive processes and behaviours, accompanied by endocrine and neural changes. In HVC—a vocal system nucleus involved in vocal perception, learning, and production—rates of neurogenesis change across seasons. However, naturally occurring rates of neurogenesis in HVC and two auditory perceptual regions—the caudomedial mesopallium (CMM) and the caudomedial nidopallium (NCM)—have not been studied in starlings. We found that females had a higher number of DCX-labeled multipolar cells in HVC compared to males, regardless of seasons. In contrast, males exhibited a higher number of these cells during fall compared to spring, suggesting that in males,

neuroplasticity may support the integration of new song elements after the breeding season. In female birds, the behavioural correlates are not as clear. However, the stable rates of neurogenesis observed here suggest that females may rely more consistently on auditory processes for conspecific discrimination, mate selection, or same-sex bonding after the breeding season. We also found that birds had more DCX-ir fusiform cells in NCM than CMM, regardless of season, indicating that neurogenesis may facilitate ongoing learning and discrimination of acoustic stimuli in natural contexts. Nonetheless, more precise behaviour measurements are required to fully understand the role of neuroplasticity in mediating seasonal behavioural plasticity in starlings.

5.3.1 Limitations

When examining the influence of sex and the environment on behavioural and neural plasticity, songbirds are often examined between categories of independent variables, like male/female, breeding/non-breeding, or season. However, these types of study designs do not account for the precise behavioural correlates of hormonal, neurobiological, and epigenetic measures. For example, when examining subjects categorized by season, it is often assumed that said subjects are engaging in seasonally appropriate behaviours, like guarding nests or sitting on eggs during breeding season. However, this assumption comes at the cost of including subjects in analyses that are not engaging in said behaviours, and thus, requires a greater reliance on speculation when interpreting results.

5.3.2 Future Directions

Applying a neuroethological approach where the behaviour of individual subjects is monitored would enable more precise interpretation of correlations and help build more predictive theories about these complex relationships. Frequency and quality of vocalization, nesting status, and contact with conspecifics are all factors that, if measured, could lead to valuable insights about the role of neuroplasticity in songbird behaviour. Logistically, such designs would be difficult to achieve in purely naturalistic settings, but they could be pursued in outdoor aviaries. For example, opportunities for different types of social interactions could be influenced by manipulating the arrangement and density of nest boxes, and within a controlled area, aspects of individual birds' vocalizations could be quantified. Such designs would help disentangle the physical aspects of environmental change from the performance of seasonally appropriate adaptive behaviours.

5.4 Conclusion

This research has provided valuable insights into the neurobiological correlates of behavioural plasticity in songbird species. The first study highlighted regional differences in DNA methylation patterns in the songbird brain, specifically between CMM and NCM, which are associated with auditory memory and discrimination. These findings suggest that DNA methylation plays a role in shaping the distinct functions of these brain regions. The second study explored the impact of social isolation and auditory discrimination on neurogenesis, revealing complex relationships between task performance and aspects of neurogenesis. Specifically, lateralization of neuronal subtypes in HVC and NCM demonstrate the involvement of neurogenesis in auditory discrimination. The third study investigated seasonal changes in neurogenesis rates, uncovering variations in neurogenic

processes and stress levels in different seasons. These findings shed light on the dynamic interplay between neurogenesis, seasonal behaviours, and stress in free-living songbirds. Overall, this research provides a foundation for future investigations into the neurobiological mechanisms underlying behavioural plasticity and highlights the need for further studies exploring the molecular and cellular processes involved in these phenomena. By investigating behavioural and neural plasticity in songbirds, we can gain a deeper understanding of the fundamental principles of how the brain adapts to environmental challenges.

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