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Sex and Seasonal Differences in Cognition and the Brain in Brood-parasitic Brown-headed Cowbirds (*Molothrus ater*)

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A thesis submitted in partial fulfillment of the requirements for the degree in Doctor of Philosophy

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SEX AND SEASONAL DIFFERENCES IN COGNITION AND THE BRAIN IN
BROOD-PARASITIC BROWN-HEADED COWBIRDS (*MOLOTHRUS ATER*)

Thesis format: Integrated Article

by

Mélanie F Guigueno

Graduate Program in Biology

A thesis submitted in partial fulfillment
Of the requirements for the degree of
Doctor of Philosophy

The School of Graduate and Postdoctoral Studies
The University of Western Ontario
London, Ontario, Canada

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Abstract

The hypothesis underlying all of neuroecology proposes that natural selection can modify cognition and its neural mechanisms if these modifications enhance fitness. I tested for sex and seasonal differences in cognition and the brain of brood-parasitic brown-headed cowbirds (*Molothrus ater*) and the closely related non-brood-parasitic red-winged blackbird (*Agelaius phoeniceus*) to determine whether cognitive and neural patterns were associated with space use and singing in the wild. Cowbirds show a reversal of sex-typical space use often seen in mammals with only female cowbirds parasitizing, searching for, and revisiting host nests. Cowbird and blackbird males sing more than females, especially in breeding condition, but female blackbirds sing, whereas female cowbirds do not sing at all. I tested cowbirds on a foraging task that required them to move through the testing environment in Chapter 2 and stationary spatial and colour memory touchscreen tasks in Chapter 3. I then examined sex and seasonal differences in the brain regions involved in spatial memory, the hippocampus, (Chapter 4) and singing behaviour, the HVC (proper name) and the robust nucleus of the arcopallium (RA), (Chapter 5) of cowbirds and blackbirds. Female cowbirds outperformed males on the foraging task and female cowbirds and blackbirds had a larger hippocampus relative to the telencephalon than male cowbirds and blackbirds, regardless of breeding condition. Female cowbirds had higher doublecortin immunoreactivity (DCX+), a measure of neurogenesis, in the hippocampus than male cowbirds, but no sex difference existed in blackbirds. However, male cowbirds outperformed female cowbirds on the spatial touchscreen task, demonstrating that females have enhanced spatial memory only on

tasks resembling their behaviour in the wild. Male and female cowbirds performed better on the spatial touchscreen task than on the colour touchscreen task, suggesting that cowbirds may have enhanced spatial memory relative to other forms of memory. Indeed, cowbirds had a larger hippocampus with higher DCX+ than blackbirds. Finally, the size of HVC and RA were positively associated with singing and DCX+ in HVC was negatively associated with singing. In conclusion, my results support the central tenet of neuroecology, namely that the brain and cognition are specialized for an organism's ecology.

Keywords: brood parasitism, brown-headed cowbird, hippocampus size, hippocampal neurogenesis, *Molothrus ater*, navigation, operant conditioning, sex differences, seasonal differences, song control nuclei, spatial memory

Co-Authorship Statement

I wrote Chapter 1 and it is not published.

A version of Chapter 2 has been published: Guigueno, M. F., Snow, D. A., MacDougall-Shackleton, S. A., & Sherry, D. F. (2014). Female cowbirds have more accurate spatial memory than males. *Biology Letters*, 10, 20140026. Danielle Snow helped collect the data and Danielle Snow, Scott MacDougall-Shackleton, and David Sherry were involved in the conceptual development, experimental design, and editing of the manuscript. Data from the 1 h retention interval in non-breeding condition were collected as part of Danielle Snow's Honours' thesis.

A version of Chapter 3 has been accepted for publication: Guigueno, M. F., MacDougall-Shackleton, S. A., & Sherry, D. F. Sex differences in spatial memory in brown-headed cowbirds: males outperform females on a touchscreen task. *PloS One*. David Sherry and Scott MacDougall-Shackleton are co-authors as they were involved in the conceptual development, experimental design, and editing of the manuscript.

Chapter 4 and Chapter 5 have not been published. I wrote these chapters and performed all the statistical analyses. David Sherry and Scott MacDougall-Shackleton will be co-authors when these chapters are published as they were involved in the conceptual development, experimental design, and editing of the manuscript.

I wrote Chapter 6 and it is not published.

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Chapter 1

1. General introduction¹

The main objectives of this thesis were to examine sex and seasonal differences in 1) spatial memory of brood-parasitic brown-headed cowbirds (*Molothrus ater*), 2) hippocampus of cowbirds and a closely-related non-parasitic relative, red-winged blackbirds (*Agelaius phoeniceus*), and 3) song control nuclei of cowbirds and blackbirds. I will first provide an overview of spatial memory and its influence on fitness, followed by an introduction to the field of neuroecology. Next, I will present a brief literature review of sex and seasonal differences in spatial memory and the hippocampus of birds and mammals. I will then summarize the ecology of obligate brood parasites and previous findings on their spatial cognition and hippocampus. Finally, I will explain why sex and seasonal differences in the singing rate of cowbirds and red-winged blackbirds are predicted to reflect differences in the song control nuclei. I will finish with detailed thesis objectives.

1.1 The importance of spatial memory

Natural selection can result in the evolution of traits that influence survival and reproduction. Spatial memory, an often neglected trait in biology, can determine whether an individual will survive and reproduce (Sherry 2006, Roth and Pravosudov 2009).

¹ This chapter has not been published.

Food-storing birds must remember the location of their cache sites to survive in harsh climatic conditions and brood parasites must remember the location of suitable host nests in which to lay their eggs (Sherry 2006). Presumably, Clark's nutcrackers (*Nucifraga columbiana*) that forgot the location of their seed caches succumbed during the frigid alpine winters and female cowbirds that forgot the location of suitable host nests in the darkness of the brief pre-dawn laying period were unsuccessful at reproducing. In both cases, these individuals with a weaker spatial memory would have failed to meet their potential for passing their genes on to the next generation, creating selection for individuals with enhanced memory. In other words, memory may have evolved to enhance fitness and thus, information that is relevant to reproduction and/or survival may be better retained (Nairne and Pandeirada 2008). Unfortunately, the mechanisms by which selection affects memory remain unclear.

1.2 Neuroecology

According to DF Sherry (2006, p. 168), "Neuroecology is the study of adaptive variation in cognition and the brain." The underlying hypothesis in neuroecology proposes that natural or sexual selection can modify cognition and its underlying neural mechanisms if these modifications enhance survival or reproduction (i.e., fitness; Rozin and Kalat 1971, Krebs et al. 1989, Sherry et al. 1989). Neuroecology makes extensive use of the comparative method (Sherry 2006). Frequently, a closely-related species that does not display the trait of interest is included in the study, along with the species of interest (e.g., Jacobs et al. 1990, Brodbeck and Shettleworth 1995, Hoshoooley and Sherry 2007). Garland and Adolph (1994) critique such two-species comparisons as lacking statistical

power because the sample size is essentially two; any two species are liable to vary in virtually any physiological trait and there is a 50% chance that the variation will occur in the predicted direction. Ideally, at least three species should be included to determine whether the trait is the ancestral or derived state (Garland and Adolph 1994 for more details); a study should include the species of interest, a closely-related species without the trait, and a sister species to both taxa so that the state of the common ancestor of the first two species can be inferred. The state of the common ancestor is essential to infer that variation in the physiological system that occurs in parallel with the trait is derived rather than ancestral. Indeed, Garland and Adolph (1994) argue that the true sample size for any such study is the number of species and any such comparison should include numerous species analyzed with a robust phylogeny. However, it is rarely logistically feasible to incorporate multiple species within a single study on neuroecology. To provide broad support for a neuroecological hypothesis, it is necessary to compare multiple studies and it is only with consistent results across species that one can conclude cognition and the brain are adapted for a specific behaviour in the wild (Sherry 2006).

Spatial memory in food-storing birds is the most extensively studied subject in neuroecology. The hippocampus is a brain region important for spatial memory, as hippocampus lesions cause a decline in spatial memory but not colour memory (Sherry and Vaccarino 1989, Hampton and Shettleworth 1996) and memory for cache sites is hippocampus-dependent (Sherry and Hoshooley 2010). Many phylogenetic analyses across a large number of bird families have supported the hypothesis that food-storing produced an enlargement of the hippocampus (Krebs et al. 1989, Sherry et al. 1989, Garamszegi and Eens 2004, Lucas et al. 2004). Pravosudov and Clayton (2002)

introduced the Adaptive Specialization Hypothesis (ASH), which states that food-storing animals should have a larger hippocampus, relative to overall brain size, than non-caching relatives and outperform these relatives on spatial memory tests, whereas no difference should exist on non-spatial memory tests. Pravosudov and Clayton (2002) only related the ASH to food-storers, although presumably the ASH could be applied to a variety of systems, including systems in which sexual selection can drive variation in cognition and the brain (see the vole examples below). The ASH is poorly defined and this nomenclature is not frequently used by other neuroecologists.

Recently, hippocampal neurogenesis, in addition to hippocampal volume, has been found to correlate with space use. In birds, hippocampal neurogenesis is a process during which new neurons are generated by mitosis in the subventricular zone and migrate into the hippocampus where they may mature and become integrated into functional circuits (Sherry and Hoshooley 2010). Individuals who rely more on spatial memory have more hippocampal neurogenesis, such as food-storing (Hoshooley and Sherry 2007) and migratory (LaDage et al. 2011) birds. In addition, food-storing in chickadees is correlated with the recruitment of new neurons into the hippocampus and neural recruitment is enhanced when chickadees are given the opportunity to store food (Patel et al. 1997). Hippocampal neurogenesis may allow individuals to encode new information about their surroundings and may help accommodate a greater spatial memory load (Barnea and Pravosudov 2011), although support for this hypothesis is lacking.

Space use in the wild may also influence cognition. Because seeds are difficult to find at some times of the year, such as winter when snow covers the ground, many seed-

eating birds store their food in caches that they then consume at a later date. These species use spatial memory to re-locate stored food. For instance, some corvids create thousands of caches in the autumn and then relocate these caches throughout the winter. Clark's nutcrackers recovered caches better, performed better on a modified radial-arm maze and performed better at a spatial memory task in an operant chamber than non-caching western scrub-jays (*Aphelocoma californica*) (Balda and Kamil 1989, Kamil et al. 1994, Olson 1991). Furthermore, food-storing nutcrackers performed better than non-food-storing scrub jays and Mexican jays (*Aphelocoma wollweberi*) on a spatial task, but not a colour task, demonstrating that selection in storing corvids has favoured spatial memory, and is not the consequence of a superior more generalized memory (Olson et al. 1995). Similarly, short-term food-storers, such as coal tits (*Parus ater*) performed better than closely-related non-food-storing great tits (*P. major*) and blue tits (*P. caeruleus*) (McGregor and Healy 1999). Another short-term food-storer, the black-capped chickadee (*Poecile atricapillus*) performed better on a spatial touchscreen task than on a colour touchscreen task whereas non-food-storing dark-eyed juncos (*Junco hyemalis*) performed equally well on both tasks (Brodbeck and Shettleworth 1995). In sum, long-term and short-term food-storers have better spatial memory than non-food-storers and have enhanced spatial memory relative to other types of memory.

Individuals within a species may show differences in cognition and the brain, based on their ecology. For example, chickadees from a harsher climate that presumably rely more on stored food have a larger hippocampus with more neurons, and perform better on a spatial task than individuals from milder climates (Roth and Pravosudov 2009). In addition, chickadees from harsher climates have higher hippocampal

neurogenesis (Chancellor et al. 2011). Thus, differences in spatial memory load in the wild can produce differences in cognition and the brain between species, but also within a species.

In sum, short-term and long-term food-storers consistently have a larger hippocampus and perform better on a variety of spatial memory tasks than non-food-storers. Food-storers also have higher levels of neurogenesis in their hippocampus. These differences in spatial memory and hippocampus volume and neurogenesis are also observed within a food-storing species with individuals varying in reliance on stored food. These findings support the hypothesis that a heavy memory load in the wild is accompanied by an evolutionary enlargement of the hippocampus relative to the size of the telencephalon and enhanced spatial memory relative to other types of memory (Krebs et al. 1989, Sherry et al. 1989, Brodin and Lundborg 2003, Sherry 2006).

Neuroecology studies normally incorporate non-model organisms because model organisms, such as fruit flies (*Drosophila* spp.), laboratory mice (*Mus musculus*), and laboratory rats (*Rattus norvegicus*) have recently undergone artificial selection by humans to facilitate laboratory based experiments. Regardless, mice and rats have a hippocampus that is anatomically homologous to the avian hippocampus, although it is located deep within the brain as opposed to the surface of the brain like in birds (Colombo and Broadbent 2000, Jarvis et al. 2005). The rodent hippocampus plays an important role in spatial memory and orientation and lesions of the rodent hippocampus disrupts performance on spatial memory tasks (reviewed in Morellini 2013). Arthropods and annelids, such as fruit flies, do not have a hippocampus, but they do possess a specialized brain region for learning and memory. The mushroom body is responsible for

olfactory learning and memory and receives olfactory information from the antennal lobe (Strausfeld et al. 1998). Thus, the mushroom body, like the hippocampus of birds and mammals, provides organisms with the ability to execute voluntary actions in response to inputs from their environment. Mushroom body size varies across species and may reflect adaptive specialization, analogous to variation in Hp with respect to food storing (Strausfeld et al. 1998). In conclusion, specialized brain regions in a variety of taxa influence cognitive processes, which in turn influences survival of these organisms.

1.3 Sex differences in spatial memory and the hippocampus

Sex differences in space use are associated with differences in spatial memory and the hippocampus. Studies examining sex differences in space use have primarily been conducted on mammals, as most mammals are polygynous and a sex difference in home ranges may lead to a sex difference in space use (Sherry et al. 1992). For example, meadow voles (*Microtus pennsylvanicus*) are polygynous, with males having home ranges 4-7 times larger than females, but pine voles (*Microtus pinetorum*) are monogamous, with no sex difference in home range size (Gaulin and FitzGerald 1986, 1989). Consistent with this difference in home range size, male meadow voles have a larger hippocampus than females and perform better on a spatial memory test, whereas no sex differences exist in pine voles (Gaulin and FitzGerald 1986, 1989, Jacobs et al. 1990). Female meadow voles have higher levels of neurogenesis in the dentate gyrus, the part of the hippocampus with the highest levels of adult neurogenesis in mammals, although this

sex difference is dependent on breeding condition (Galea and McEwen 1999). However, male Richardson's ground squirrel (*Urocitellus richardsonii*), which are also polygynous, have a larger relative hippocampus with more neurogenesis than females (Burger et al. 2013, Burger et al. 2014). Thus, in mammals, the sex with the larger home range (usually males) also has the larger hippocampus, but patterns of neurogenesis relative to home range size are not as consistent (Galea and McEwen 1999, Sherry et al. 1992, Burger et al. 2014).

Sex differences in spatial memory can be specific to the type of task. In humans, males outperform females on mental rotation and navigational tasks but females outperform males on stationary spatial working memory tasks involving the memory for the location of objects in a spatial array (Voyer et al. 1995, Silverman et al. 2000, Postma et al. 2004, Voyer et al. 2007, Hampson 2008). Mental rotation tasks require subjects to visualize three-dimensional images whereas spatial working memory refers to temporary storage and processing of spatial information (Hampson 2002).

Like in mammals, sex differences in spatial memory exist in birds. Male hummingbirds (*Seiurus aurocapillus*) are better than females at remembering the location of high quality nectar sources (González-Gómez et al. 2014). However, unlike mammals, 90% of birds are socially monogamous and provide bi-parental care (Mock and Fujioka 1990). As such, we would expect sex differences in space use and spatial memory to be more frequent in mammals than in birds, although research in this field of study is lacking.

1.4 Seasonal changes in spatial memory and the hippocampus

Breeding condition can influence spatial memory. Changes in breeding condition affect circulating levels of testosterone, which in turn can influence learning of spatial tasks and performance on these tasks (Oberlander et al. 2004, Hodgson et al. 2008, Bailey et al. 2013). The songbird hippocampus expresses high levels of aromatase, resulting in high levels of local estrogen synthesis from testosterone and enhanced spatial memory acquisition and performance (Oberlander et al. 2004, Saldanha et al. 2004, Bailey et al. 2013). Thus, testosterone, which increases in breeding condition, can enhance spatial memory in songbirds.

Although breeding condition can influence spatial memory, there are no consistent seasonal patterns in hippocampus size or neurogenesis. One study showed that hippocampus size increased by 40% in the autumn in chickadees, relative to the spring, and corresponded to the first half of the food-storing season (Smulders et al. 1995). However, subsequent studies were unable to replicate these results and one study actually reported a larger hippocampus from February to April (Hoshooley and Sherry 2007). Two more studies found no change in hippocampal volume throughout the year (Hoshooley and Sherry 2004, Hoshooley et al. 2007). In support of Smulders et al. (1995), Barnea and Nottebohm (1994) reported that hippocampal neurogenesis was also at its peak in the autumn, but Hoshooley and Sherry (2004) found no seasonal variation in the production of new neurons and concluded that results from Barnea and Nottebohm (1994) could only be explained by seasonal differences in the survival of new neurons.

Thus, the seasonality of total size and recruitment of new neurons into the hippocampus varies among studies, suggesting that food-storing birds may respond to annual changes in food storing itself, which is in turn influenced by factors such as food availability, energy balance, and flock dominance structure (Sherry and MacDougall-Shackleton 2014).

Breeding condition has also been reported to influence spatial memory in mammals. Male meadow voles and deer mice show better spatial learning than females in breeding condition only (Galea et al. 1995, 1996). Depending on the task, natural fluctuations in estrogen levels in female humans can either increase or decrease spatial memory. For example, females perform better on mental rotation tasks during the menstrual phase of their menstrual cycle, when estrogen levels are at their lowest (Hampson 2002). However, high levels of estrogen are associated with better performance on object location memory tasks (Hampson and Morley 2013).

Hormones, which change with breeding conditions in mammals, also influence the hippocampus. Male meadow voles with high levels of testosterone, which simulated breeding condition, had a larger hippocampus than females with low estradiol levels, whereas that difference disappeared in male voles with low levels of testosterone (Galea et al. 1999). In addition, wild and laboratory female meadow voles had the highest levels of neurogenesis in their dentate gyrus in the non-breeding season and these high levels of neurogenesis were associated with lower levels of corticosterone and estradiol (Galea and McEwen 1999, Ormerod and Galea 2001, Galea et al. 2013). Richardson's ground squirrels are polygynous but males had a larger hippocampus in the non-breeding season, which may reflect male-only caching in the non-breeding season (Burger et al. 2013). In

a follow up study, Burger et al. (2014) reported that hippocampal neurogenesis was highest in the non-breeding condition and that the dentate gyrus was larger in the breeding season when males are mating with multiple females within their larger home range. Thus, the extent to which these hippocampal changes reflect space use in ground squirrels is unclear, but could reflect a combination of seasonal changes in caching behaviour and mating (Burger et al. 2013, 2014).

How do hormones affect behaviour and the hippocampus? In rodents, elevated levels of estradiol, which can be produced from testosterone via the enzyme aromatase, increase the number of dendritic spines and the number of synapses onto these spines in hippocampal neurons (Woolley and McEwen 1994, Murphy and Segal 1996, Yankova et al. 2001). In rodents, estrogens enhance synaptic transmission in the hippocampus and improve spatial memory performance (Gibbs 1998, Woolley et al. 1997). Less is known about the effect of hormones on the avian hippocampus, but several studies have shown that aromatase is enriched in the hippocampus in several species of songbirds (Shen et al. 1994, Saldanha et al. 1998, Metzdorf et al. 1999, Fusani et al. 2000). Aromatization of testosterone produces high levels of estradiol in the hippocampus which likely maintains the integrity and function of circuits within the hippocampus. Indeed, the songbird hippocampus expresses high levels of estrogen and androgen receptors, providing a direct site for sex steroids to act on hippocampal function, including spatial memory (Hodgson et al. 2008).

1.5 Avian brain nomenclature

Some birds are just as intelligent as some mammals and even rival the great apes in many cognitive tasks (Emery 2006). For example, some bird species are known to have episodic-like memory, tool use, the ability to pass knowledge via social learning, the ability to learn vocally, or high capacity for learning-based sound localization (Jarvis et al. 2005, Emery 2006). Nonetheless, conventional nomenclature for the avian brain was developed by Ludwig Edinger in the 19th century based on the belief that mammalian brain morphology was evolutionary newer and more sophisticated. Edinger used the suffix “striatum” to describe regions of the brain that were associated with basal ganglia in mammals, and applied those terms to the avian cerebrum. Jarvis et al. (2005) proposed renaming bird brain morphology to portray birds as more comparable to mammals in their cognitive ability as we now know that supposedly primitive regions of bird brains are comparable in their genetic and biochemical machinery to mammals and capable of complex neural processes homologous to regions in mammals (Jarvis et al. 2005). Indeed, the avian and mammalian hippocampi are homologous (Colombo and Broadbent 2000, Jarvis et al. 2005). Realization of avian intelligence has been an important part of recent research and has inspired studies like this thesis to integrate the study of cognition and the brain with other fields, such as behavioural ecology.

1.6 Obligate brood parasites

1.6.1 Ecology and phylogeny

Brown-headed cowbirds (*Molothrus ater*) are ideal for testing the adaptive specialization of memory, as they experience both sex and seasonal differences in spatial memory load. Brown-headed cowbirds and red-winged blackbirds, the non-parasitic control species used in my thesis, shared a common ancestor less than 5 million years ago (Sorenson and Payne 2002, Mermoz and Ornelas 2004, Kruger 2007). This most recent non-parasitic ancestor gave rise to two icterid groups: the *Molothrus* spp., which includes brown-headed cowbirds and all other cowbird species, and the *Agelaius* spp., which includes red-winged blackbirds. Therefore, brown-headed cowbirds and red-winged blackbirds are evolutionary close enough that potential differences in cognition and the brain could be linked to differences in behaviour rather than divergent phylogeny.

Brown-headed cowbirds are obligate brood parasites that rely on approximately 150 bird species to raise their young (Davies 2000). Only female cowbirds search for the nests of these host species in the breeding season. Female cowbirds parasitize nests during a brief one-hour window before sunrise when it is still dark, so they must find a suitable host nest at least one day before parasitism (Rothstein et al. 1986). In addition to finding these nests, females must monitor their nesting stages to ensure that parasitism occurs by early incubation, allowing enough incubation time for the cowbird eggs to hatch (White et al. 2009). Female cowbirds will also revisit some nests they have parasitized, or they are about to parasitize, to remove a host egg (Sealy 1992, Guigueno and Sealy 2011). Thus, female cowbirds make multiple visits to a given host nest and are

under strong selection to remember the locations of these nests and their nesting stage to increase the probability that their eggs hatch and that their young are successfully raised by the hosts (Rothstein et al. 1986, Gates and Evans 1998, White et al. 2009).

A long history of field research on brown-headed cowbirds has shown that their mating system varies among populations and can be monogamous, promiscuous, polygynous, or polyandrous (Lowther 1993). The cowbird mating system may be influenced by population density and sex ratio, with monogamy occurring most frequently in low density populations when males can easily attend mates (Lowther 1993). Although variable among populations, monogamy with promiscuity is the most frequently documented mating systems in cowbirds (Yokel 1986, Yokel 1989, Maguire et al. 2013). At the beginning of the breeding season, females establish large breeding territories based on the location of host nest sites and males engage in a competitive scramble for reproductive opportunities (Rothstein et al. 1984). Each male identifies a receptive female and sings intensively to her (West et al. 1981, Rothstein et al. 1986). Individual males will follow their female in their host nest territory in the morning to mate-guard (Davies 2000). This monogamous mating system can become promiscuous for two main reasons. First, freed from parental care, females can become serial monogamists over the course of the breeding season. Second, males may mate with other females if host nest territories overlap (Davies 2000). In the afternoon, both female and male cowbirds leave the forested host nest territories and travel, sometimes several kilometres, to short-grass feeding areas (Rothstein et al. 1984). In sum, female cowbirds show a pattern of space use requiring a heavier memory load than males, with only females parasitizing host nests before sunrise when it is dark (Rothstein et al. 1986) and

females leading host nest visitations in the morning (Davies 2000). Thus, cowbirds are a rare example of a species demonstrating a reversal of typical sex-differences in spatial memory load (Sherry et al. 1992).

1.6.2 Brain

Associated with sex differences in spatial memory load, female brown-headed cowbirds were found to have a larger hippocampus relative to males in the breeding season in one study (Sherry et al. 1993), but not another (Lattanzio 2007). A female-biased sex difference in hippocampus size was also reported in shiny cowbirds (*M. bonariensis*), a South American relative in which, like brown-headed cowbirds, only females search for nests (Reboreda et al. 1996). In screaming cowbirds (*M. rufoaxillaris*), males assist in nest searching and no sex difference in hippocampus size was observed (Reboreda et al. 1996).

The hippocampus in multiple cowbird species varies seasonally. Clayton et al. (1997) found that South American shiny and screaming cowbirds had a larger hippocampus in the breeding season relative to the non-breeding season and that female shiny cowbirds had a larger hippocampus than males. Additionally, previous work reported that female brown-headed cowbirds had a larger hippocampus early in the breeding season (April), when nest searching is frequent, than later in the summer (July), after breeding has ended, whereas male hippocampus size did not change seasonally (Lattanzio 2007). Similarly, the number of new neurons recruited into the hippocampus was greater in April than July in females, but no seasonal difference occurred in males, suggesting that neurogenesis facilitated a higher spatial memory load in females.

Lattanzio (2007) did not include a non-brood-parasitic relative in her study, which is an important component of the comparative method in neuroecology (see above). In sum, the hippocampus of multiple cowbird species seem to vary seasonally, with a larger hippocampus during the breeding season, although only two studies have found seasonal differences in hippocampus size. No researcher has made a connection between seasonal change in hippocampus size and cognition.

1.6.3 Cognition

I am not aware of any previous study addressing seasonal changes in cognition in any brood parasite. Some studies examining sex differences in cognition have generated puzzling results.

No sex difference was recorded in shiny cowbirds in an experiment testing the speed of food recovery when food rewards were associated with specific locations or colours (Astié et al. 1998). Females but not males, however, learned to recover food faster when it was associated with a colour (Astié et al. 1998). Therefore, no sex difference was reported for the spatial test in which a sex difference was expected, but a sex difference was reported for the non-spatial test in which no difference was expected.

In a previous touchscreen experiment, female brown-headed cowbirds outperformed males on a touchscreen spatial memory task, whereas both sexes performed equally well on a colour memory task (delayed-matching-to-sample; Lattanzio 2007). However, a few issues arose during this study. First, cowbirds performed just over random performance, suggesting that the cowbirds may have had difficulty learning the task. Also, cowbirds would periodically cease responding in the middle of a trial, leading

to less than 20 total trials in a session (Lattanzio 2007), suggesting they were not motivated to complete a large number of trials. Typically, sessions of at least 20 trials are considered sufficient (Hodgson et al. 2008). Cowbirds were not tested seven days a week which could have played a role in them performing poorly and completing a low number of trials per session. Finally, the effect of breeding condition on spatial memory was not tested in this study (Lattanzio 2007). Therefore, consistent patterns of sex and seasonal changes in the hippocampus and spatial memory of cowbirds have yet to be found.

1.7 The song control nuclei

The focus of my thesis was on spatial memory and the hippocampus, the neural control for spatial memory. However, I also applied the principles of neuroecology to examine whether differences in the ecology of icterids—specifically different use of song between sexes, seasons and species—lead to differences in the song control nuclei. The nuclei of interest, HVC (proper name) and the nucleus robust nucleus of the arcopallium (RA), are important for song production in songbirds (Nottebohm et al. 1976) and likely also for song learning and perception, although this latter function is unclear (Scharff and Nottebohm 1991). Sex and seasonal patterns of HVC and RA are more consistent across studies and species than with the hippocampus. Male songbirds, who generally sing more than females, have a larger HVC and RA than females (reviewed in MacDougall-Shackleton and Ball 1999). HVC and RA are also larger in breeding males and this pattern is associated with a seasonally higher rate of singing (Tramontin and Brenowitz 2000). Neurogenesis levels are highest in non-breeding in HVC, when birds are practicing and modifying their song for the upcoming breeding season (Tramontin and

Brenowitz 2000). There is no neurogenesis in RA as neuron numbers do not change seasonally (Tramontin and Brenowitz 2000, Wada et al. 2014).

Seasonal changes in HVC and RA growth are driven in part by testosterone (T), although the physiological mechanisms underlying these seasonal changes are not fully understood. In breeding condition, song stereotypy and song rate are at the highest, when plasma T concentrations are also high, whereas song stereotypy, song rate, and plasma T are lowest in non-breeding condition (Wingfield and Farner 1978, Smith et al. 1997). Administration of exogenous T to castrated and non-breeding males induces growth of song control nuclei and to female canaries induces singing and the growth of song control nuclei (Nottebohm 1980, Bernard and Ball 1995, Smith et al. 1997, Strand and Deviche 2007). T can be converted, irreversibly, to dihydrotestosterone (DHT) and 17β -estradiol (E2) by the enzymes 5α -reductase and aromatase, respectively, in the brain of songbirds, which expresses both of these enzymes (Soma et al. 2003). DHT and E2 then bind to androgen and estrogen receptors, which are expressed in the HVC and RA (Harding et al. 1984, Gahr et al. 1993, Kolvenbag et al. 1998, Soma et al. 1999). T can also bind to androgen receptors, but with 100-200 times less affinity (Kolvenbag et al. 1998). Administration of DHT, E2, and DHT+E2 to castrated white-crowned sparrows increases the volumes of HVC and RA as much as the administration of T (Tramontin et al. 2003). Therefore, the effects of T on the seasonal plasticity of HVC and RA are likely mediated by DHT and E2, agonists for androgen and estrogen receptors, respectively.

Neurogenesis in the HVC plays an important role in the seasonal growth of this song control nucleus. Binding of E2 to estrogen receptors upregulates the expression of vascular endothelial growth factor receptor-2 (VEGF-R2) in vascular endothelial cells in

HVC capillaries (Louissant et al. 2002). Binding of T to androgen receptors up-regulates the production of vascular endothelial growth factor (VEGF) in the HVC neurons (Louissant et al. 2002). Upregulating VEGF-R2 and VEGF increases the production of brain-derived neurotrophic factor, a neurotrophin that plays a direct role in the differentiation and survival of new HVC neurons (Rasika et al. 1999; see Figure 3 in Robertson et al. 2014).

Sex and seasonal differences in singing rate, which are mediated by HVC and RA, exist in my two study species, brown-headed cowbirds and red-winged blackbirds. Both male red-winged blackbirds and brown-headed cowbirds sing more than females and males sing more frequently in breeding condition than in non-breeding condition (Nero 1956, Beletsky 1983, Kirn et al. 1989, Hamilton et al. 1997, reviewed in Hall et al. 2010). However, female red-winged blackbirds do sometimes sing whereas female brown-headed cowbirds do not sing at all (Hamilton et al. 1997). In addition, because brown-headed cowbirds are brood parasites, males develop their song in winter flocks, several months after leaving the nest of their host (King and West 1988), whereas red-winged blackbirds start learning their song in the nest (Marler et al. 1972, Yasukawa et al. 1980). Both red-winged blackbirds and cowbirds are open-ended learners and their song varies year to year (Marler et al. 1972, King and West 1988). However, female choice likely plays a more important role in male song in cowbirds, as males modify their song in response to stimulation by females (King and West 1988, Hamilton et al. 1997). In blackbirds, male-male competition has a stronger influence on song repertoires than female choice (Marler et al. 1972, Yasukawa et al. 1980). Blackbirds and cowbirds,

although closely related, differ in various aspects of their song and thus are excellent candidates to study sex and seasonal changes in their song control nuclei.

1.8 Thesis objectives

My thesis is separated into two parts. The first part examines sex and seasonal differences in spatial memory in cowbirds (Chapters 2 and 3), whereas the second part examines sex and seasonal difference in the brain of cowbirds and blackbirds, namely the hippocampus (Chapter 4) and two song control nuclei, HVC and RA (Chapter 5).

My first objective was to determine whether female cowbirds are consistently better across spatial tasks and whether spatial performance differs between breeding conditions. I tested female and male cowbirds on two types of spatial tasks. The first task required subjects to find a single baited cup either 1 h or 24 h after initially being exposed to the location of the baited cup (Chapter 2). Retention intervals of 1 h and 24 h were meant to simulate female cowbirds returning to a host nest later in the morning after parasitism to remove a host egg (1 h) and females monitoring nests daily to assess whether the nesting stage is appropriate for parasitism (24 h). The second task required the birds to peck a shape on a touchscreen that was in the same location as a shape displayed 15- 60 s earlier (Chapter 3). The food cup task required subjects to move through an open spatial environment whereas the touchscreen task did not. Thus, the food cup task was more ecologically-relevant than the touchscreen task. However, the touchscreen task, unlike the food cup task, was automated, which allowed me to collect data over more trials and also test non-spatial (colour) memory as a control. For both

tasks, I varied the photophase of the photoperiod to change the subjects' breeding conditions and test peak performance in breeding and non-breeding condition.

The second part of my thesis dealt with the brain. Chapter 4 focused on the part of the brain responsible for spatial memory, the hippocampus. My goal was to determine whether sex and seasonal patterns of spatial memory measured in Chapters 2 and 3 correlated with sex and seasonal patterns of hippocampus size and neurogenesis. In addition, red-winged blackbirds were included as the non-brood-parasitic comparison species. Chapter 5 focused on two song control nuclei, HVC and RA. Although sex and seasonal differences in the size of song control nuclei have been extensively studied in a variety of songbirds, few studies have simultaneously examined sex and seasonal differences in the volume and neurogenesis of song control nuclei. In addition, my research incorporates data on cowbirds and blackbirds, two species that differ in song development and sex differences in song rates.

My thesis incorporates data on female and male cowbirds and red-winged blackbirds. I did not include a third species (a sister species to both cowbirds and red-winged blackbirds) as called for by Garland and Adolph (1994) because doing so would not have allowed me to thoroughly investigate sex and seasonal differences in cognition and the brain. Therefore, data from my thesis would need to be compared to past and future studies addressing similar questions in cowbirds to determine if observed differences are consistent and are adaptations, just like extensive research has showed that food-storing has produced an evolutionary enlargement of the hippocampus and enhanced spatial memory.

1.9 References

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Chapter 2

2. Female cowbirds have more accurate spatial memory than males²

2.1 Introduction

Memory can have profound effects on fitness and survival, but it is unclear exactly how natural selection has affected the evolution of memory. The predominant hypothesis in neuroecology, the adaptive specialization hypothesis (ASH), proposes that the brain and cognition are adaptively specialized to solve specific ecological problems (Sherry 2006, Roth and Pravosudov 2009). For example, mating systems may favour greater spatial abilities in one sex over the other. Polygynous male voles (*Microtus* spp.) have a larger home range, a larger hippocampus, and outperform females on a spatial memory task, whereas no sex differences exist in monogamous voles (Gaulin and Fitzgerald 1986, 1989, Jacobs et al. 1990).

Brown-headed cowbirds (*Molothrus ater*) provide a strong test of the ASH

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because they exhibit a sex difference in space use that is the reverse of most species, with females having a greater spatial memory load. Cowbirds are obligate generalist brood parasites. Only females locate, monitor, and revisit the host nests they parasitize; female reproductive success depends on spatial ability (Rothstein et al. 1986). Females locate host nests by searching the canopy, watching host nest building activity and attempting to flush incubating hosts from their nests (Norman and Robertson 1975). Female cowbirds, which spend their mornings in their egg-laying range either alone or followed by males, parasitize nests before sunrise when it is still dark, and must, therefore, have an accurate memory of the locations of potential host nests (Rothstein et al. 1986, Gates and Evans 1998). Female brown-headed cowbirds have a larger hippocampus than males, whereas no sex difference exists in related species that are not brood parasites (Sherry et al. 1993, Rothstein et al. 1996, Clayton et al. 1997). This difference in the hippocampus size between males and females may be present only in the breeding season (Clayton et al. 1997). Sex and seasonal differences in spatial cognition in cowbirds, however, are not well understood (Astié et al. 1998).

I tested female and male cowbirds' spatial memory in breeding and non-breeding conditions using a delayed-matching-to-sample (DMTS) spatial memory task. Birds re-located a single covered cup containing food among 25 cups after a retention interval (RI) of 1 h or 24 h. Although the rewarded cup was not a host nest and did not contain eggs, I took the ability to return to a baited cup location as a general test of memory for spatial location (Gaulin and Fitzgerald 1986). The 1 h RI mimicked the interval between laying an egg and returning to remove a host egg (Sealy 1992). The 24 h RI mimicked the interval between discovering a potential host nest, monitoring it daily, and parasitizing it

(Sealy 1995, White et al. 2009). I hypothesized that in response to the cognitive demands placed on female cowbirds by brood-parasitic breeding, females would make fewer errors than males and that the sex difference would be most pronounced in breeding condition, when females search for host nests in the wild.

2.2 Methods

2.2.1 Capture and maintenance of cowbirds

Female ($n = 7$) and male ($n = 7$) brown-headed cowbirds were captured at Queen's University Biological Station near Elgin, Ontario (5 of each sex) and at Long Point Bird Observatory near Port Rowan, Ontario (2 of each sex). After several months of habituation to captivity, I food deprived birds to maintain them at 85% of their *ad libitum* weight in captivity in order to motivate them to perform the food-rewarded spatial task. Eighty-five percent of *ad libitum* captive weight was similar to the birds' free-living body weight (i.e., body weight at capture from the wild). Birds did not have any prior experience with the apparatus or any other form of training.

2.2.2 Photoperiod manipulation

Birds were first tested in photorefractory non-breeding condition after being housed on a long day photoperiod for 5 months (16 h L: 8 h D). Following this, I maintained birds on a short-day (8 h L: 16 h D) photoperiod for 60 d to induce photosensitivity, then switched them to long days (14.5 h L: 9.5 h D) to induce photostimulated breeding condition (Dawson 2003). Following photostimulation, I re-

tested all birds using 5 practice trials and 10 testing trials for both the 1 and 24 h RI. To confirm that birds were in non-breeding and breeding condition I collected blood samples during both periods of testing.

2.2.3 Blood sampling

Blood samples were collected within 30 min of entering the housing room on days that birds were not food deprived or tested. I collected approximately 300 μ L of blood via brachial venipuncture into heparinized capillary tubes, centrifuged blood at 13,000 \times g for 10 min and stored the separated plasma at -30 $^{\circ}$ C until hormone assay.

2.2.4 Androgen assay

Plasma androgen concentration was assayed using a testosterone enzyme immunoassay previously validated for a variety of bird species including songbirds (EIA; Cat. #1-2402, Salimetrics; Washburn et al. 2007). Testosterone has been shown to increase in breeding condition for both female and male brown-headed cowbirds (Dufty and Wingfield 1986 a,b). I followed the kit directions except that plasma samples were diluted five times. To validate the assay for cowbirds I followed Newman et al. (2008) and assayed a serial dilution of cowbird plasma and compared it to the standard curve using an ANCOVA. A non-significant interaction term ($R^2 = 0.84$, no significant interaction, $F_{1,9} = 0.42$, $p = 0.54$) indicated the slopes were similar and that the assay was suitable for cowbirds. Intra-assay variation was 9.43%. Inter-plate variation, based on a pooled cowbird plasma sample and low and high controls was 9.41%. The sensitivity of our assay was 1 pg/ml (two standard deviations from the average value of zero on my

four standard curves). Samples below 1 pg/ml were assigned a value of 0.5 pg/ml for analyses.

2.2.5 Song recording and laparotomies

I also recorded song frequency in the cowbird housing room from 10:00 to 13:00 EDT in the middle of testing in non-breeding condition, three times during breeding and once after testing. Sentinel cowbirds housed in the same room as the test subjects were laparotomized during non-breeding and breeding condition testing. Birds were anesthetised with isoflurane and a small incision was made on the left flank to allow visual assessment of gonad size (MacDougall-Shackleton et al. 2006).

2.2.6 Testing

I tested cowbirds in a 180 X 180 cm wire mesh enclosure (75 cm in height) with a door on each of the four sides. During training and testing, I food-restricted subjects to maintain them at 85% of their free-feeding weight, which was similar to their free-living body weight (i.e., body weight at capture from the wild). Twenty-five cups formed a 5x5 square array on the floor with 10 cm between cups (Figure 2.1). In the sample phase of each trial, a bird entered the apparatus through one of the four doors determined at random and was then free to search the array of open cups to locate the one cup baited with millet and mealworms. I used food as a reward because males will not search for nests or eggs (Z. Torok, unpublished data). The baited cup was randomly selected on

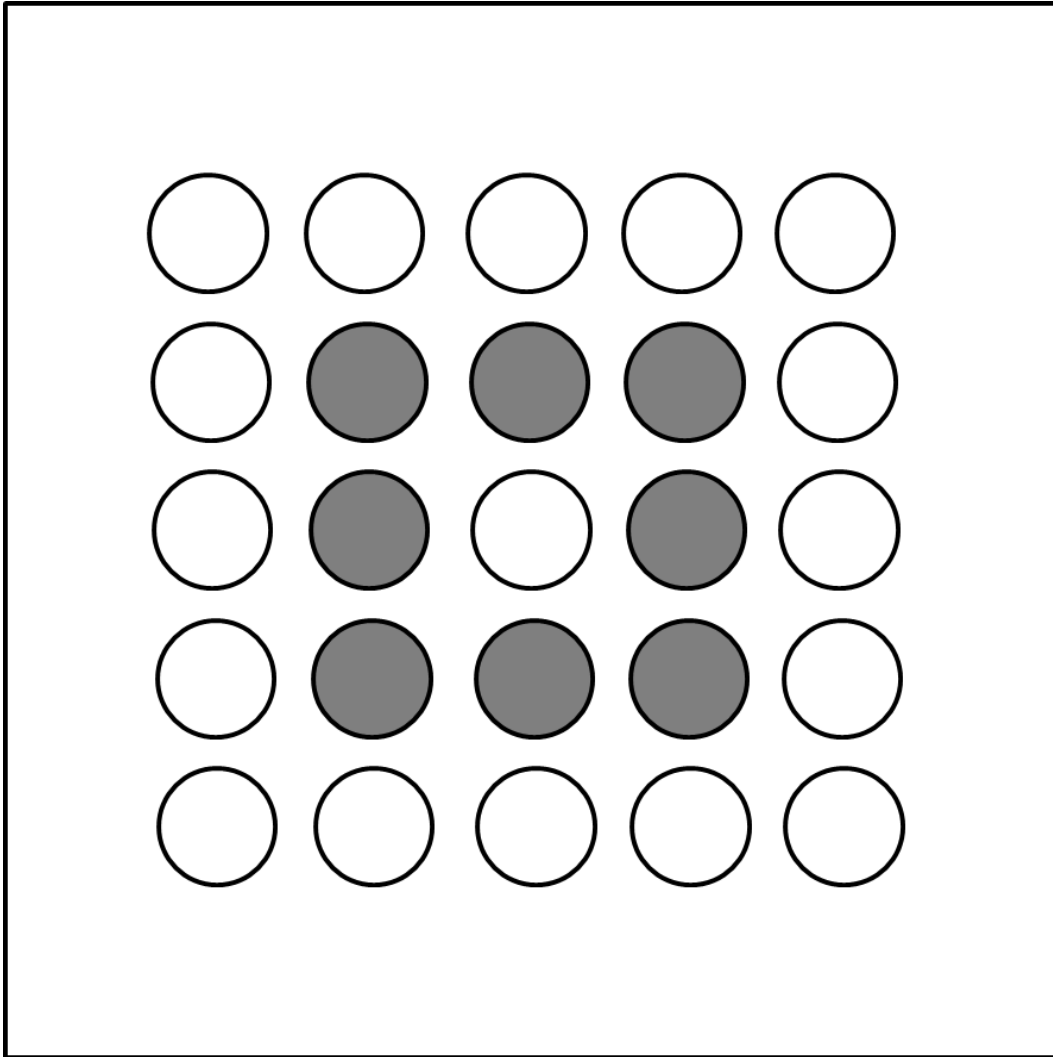


Figure 2.1. Diagram of the testing apparatus. Possible rewarded cups are shaded.

each trial to be one of the eight interior cups, excluding the centre cup (Figure 2.1). Once the food was located, the bird was permitted to eat for two minutes. Following a 1 h or 24 h RI in its home cage, the bird re-entered the apparatus for the matching phase of the trial, again through one of the four randomly assigned doors. During this phase the baited cup was in the same location, but all of the cups were individually covered with white paper lids. The bird's task was to find the cup that matched the location of the cup that was baited in the sample phase. To reduce the possible use of olfactory cues to locate the baited cup, all cups were shaken with millet inside for 15 sec. Once the food was located, the bird was given five minutes to eat the food as a reward. If a bird did not find the baited cup within 20 min, the trial was ended and scored as unsuccessful.

To assess memory in the matching phase for the location of the baited cup, I measured the number of errors before finding the baited cup and the time and path length between uncovering the first cup and uncovering the baited cup. Tortuosity of search was estimated as path length divided by the shortest possible path between entering the enclosure and the baited cup (the arc-chord ratio, equal to the inverse of the straightness index; Benhamou 2004). The expected number of errors to the first success is 25 if birds search at random and repeated visits to the same cup are scored as errors (i.e., sampling with replacement). If birds learn that only the 8 interior cups are ever baited, the expected number of errors to the first success, scoring revisits as errors, would be 8. Because birds frequently revisited cups they had already opened, and these revisits were scored as errors, these values seem the best estimates of the number of errors expected by chance. If we assume instead that birds never revisit cups they have already opened (i.e. sample without replacement), then the number of errors expected by chance, based on the

negative hypergeometric distribution, is 13 if birds searched all 25 cups and 4.5 if they searched only the interior 8 cups.

Birds were tested every day; a full 24 h RI trial required two days to complete and a 1 h RI trial, a single day. Birds were tested first in non-breeding condition with the 1 h RI followed by the 24 h RI, and then re-tested in breeding condition with the 1 h RI followed by the 24 h RI. Birds learned the task during the 1 hr RI in non-breeding condition and were given 10 practice trials before the 10 testing trials. During the 24 h RI in non-breeding condition and 1 h and 24 h RIs in breeding condition, cowbirds were given 7, 5, and 5 training trials, respectively, before 10 testing trials. Acquisition was measured as the number of errors made across trials and the proportion of individuals who successfully completed the matching phase within the 20 min trial duration.

Non-breeding and breeding conditions were induced by manipulation of photoperiod and breeding condition was verified by hormone assay and measurement of singing rates and gonads. Performance and acquisition data were analyzed using linear mixed models with PROC MIXED in SAS 9.3 (SAS Institute, Cary NC) with repeated measures for each subject across breeding conditions.

2.3 Results

2.3.1 Plasma androgens

There was a significant effect of breeding condition on androgen concentrations for females ($F_{1,6} = 35.34$, $p = 0.001$) and males ($F_{1,6} = 26.86$, $p = 0.002$), with higher androgen levels in breeding condition (Figure 2.2).

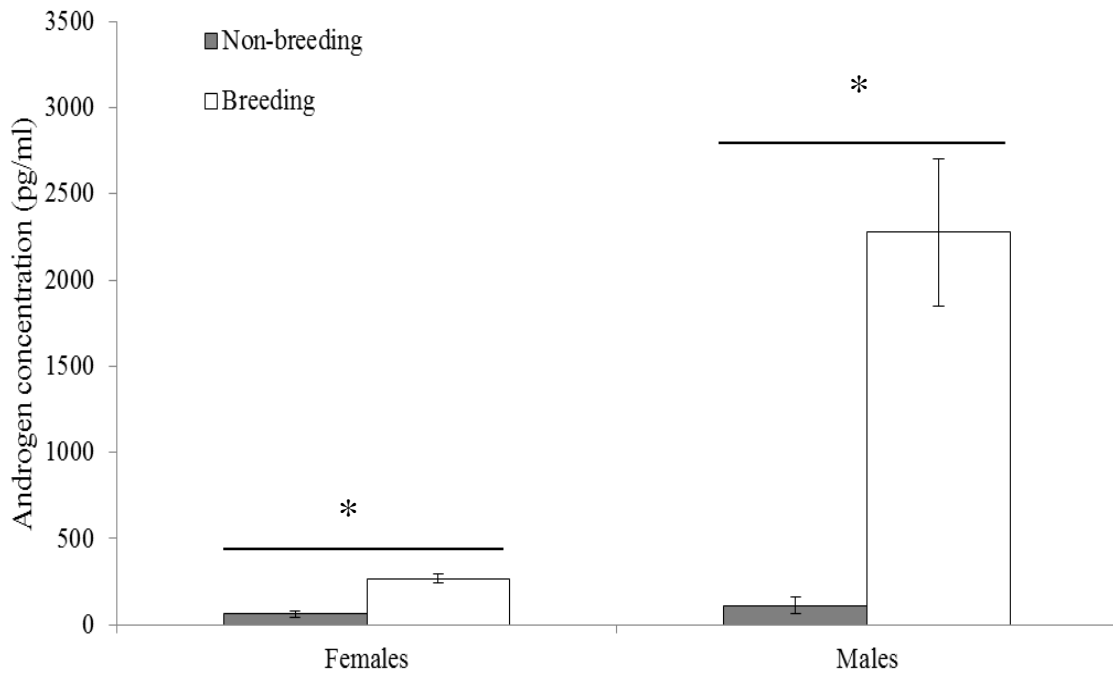


Figure 2.2 Changes in androgen levels between breeding conditions. Females and males had higher androgen levels in breeding condition than in non-breeding condition. Asterisks indicate $p \leq 0.05$.

2.3.2 Song frequency

Song frequency in non-breeding condition (147 songs per hour) was about a tenth of the frequency recorded in breeding condition (1224, 1098, and 969 songs per hour). After the testing was completed and the birds moulted, song frequency decreased to a non-breeding level (72 songs per hour).

2.3.3 Laparotomies

I performed laparotomies on sentinel birds during non-breeding and breeding conditions to record changes in gonadal development. I measured the testes of three males twice in non-breeding condition (range 1.5-2 mm) and two males twice in breeding condition (range 3-4 mm), confirming that testes had developed. The ovaries of three females examined twice in non-breeding condition were granular and showed no follicle hierarchy. I did not perform laparotomies on females in breeding condition because a change in gonadal development in females is generally only seen a few days before egg-laying (SM-S, pers. obs.). However, near the end of testing in breeding condition, three females in my colony laid eggs for one to 14 consecutive days, confirming that females also underwent gonadal development.

2.3.4 Task acquisition

Birds were trained on the 1-hr RI in non-breeding condition. There was a significant effect of session number ($F_{19,201} = 14.96$, $p < 0.0001$), but no significant effect of sex ($F_{1,12} = 0.51$, $p = 0.49$) and no sex by session interaction ($F_{19,201} = 0.25$, $p = 1.00$), with birds achieving better than chance performance (fewer than 8 errors) on trial 2 of the 1 hr

RI in non-breeding condition with no effect of sex or sex by trial interaction (Figure 2.3). Over the 20 total trials (10 practice, 10 testing trials) on the 1 h RI in non-breeding condition, there was a significant increase in success across trials ($F_{19, 219} = 1.77, p = 0.03$). There was no effect of sex ($F_{1, 12} = 0.23, p = 0.64$), or sex by trial interaction ($F_{19, 219} = 0.69, p = 0.83$). The proportion of successful individuals in the 10 testing trials did not differ among the four testing conditions (1 h and 24 h RIs, non-breeding and breeding; $F_{9, 117} = 0.67, p = 0.73$).

2.3.5 Performance

Errors across RIs and breeding conditions

Birds improved as testing progressed ($F_{3, 38} = 6.98, p = 0.0007$, Figure 2.4A). Performance in the first condition (1 h non-breeding) was significantly different from the last three conditions probably because task acquisition continued during this initial condition (Tukey's tests: 24 h non-breeding, $t_{38} = 3.56, p = 0.005$; 1 h breeding, $t_{38} = 3.59, p = 0.005$; 24 h breeding, $t_{38} = 4.03, p = 0.001$). Because performance was likely to be still improving during testing for the 1 h RI in non-breeding, the remainder of my analyses are based on the 24 h RI only.

Number of errors

There was a significant effect of sex, with females making fewer errors than males (Table 2.1, Figure 2.4B). There was no effect of breeding condition or interaction between breeding condition and sex (Table 2.1, Figure 2.4B).

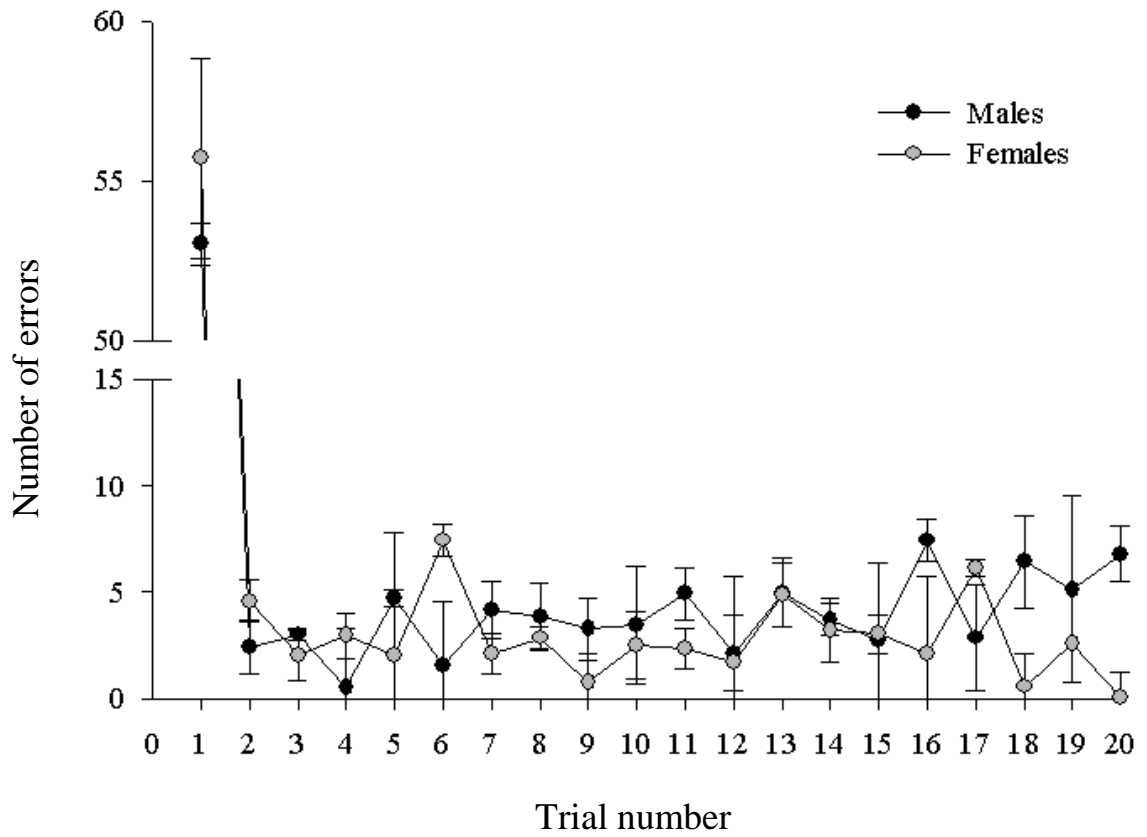


Figure 2.3 Acquisition of the spatial memory task. Cowbirds learned the task with a 1-hr RI during non-breeding condition.

Figure 2.4 **A)** Number of errors made by male and female brown-headed cowbirds combined across retention intervals (RI) and breeding conditions in testing order. Performance improved after the first condition (1 h non-breeding; asterisk indicates $p \leq 0.05$). **B)** Number of errors before rewarded cup was opened during the 24 h RI. **C)** Time required to find rewarded cup once searching commenced during the 24 h RI. **D)** Path tortuosity: length of the path taken by the bird from the enclosure entrance to the correct cup divided by the shortest possible path from the entrance to the correct cup during the 24 h RI. Females are in light grey whereas males are in dark grey (B-D). Asterisks indicate a main effect of sex (B-D). All values are means \pm SE.

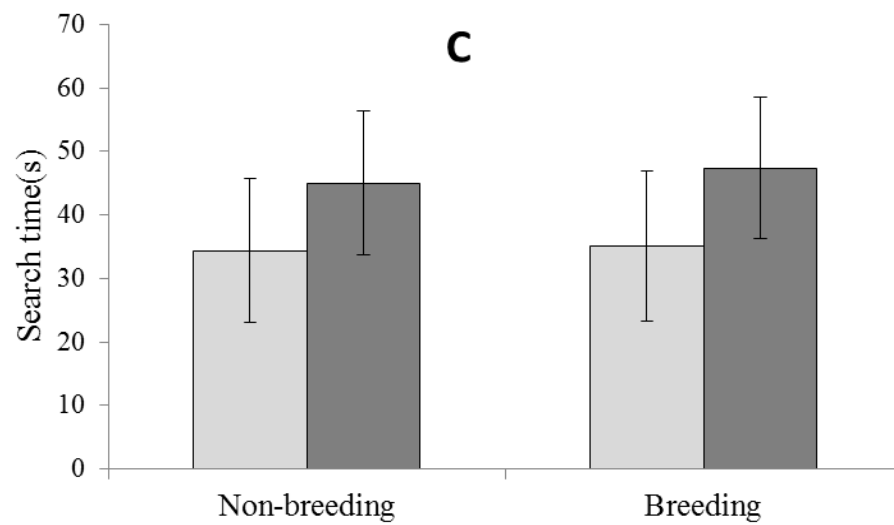
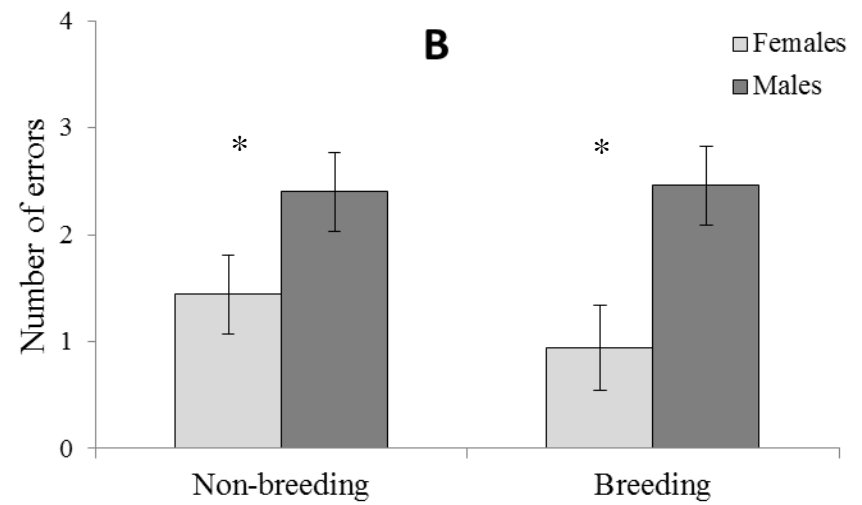
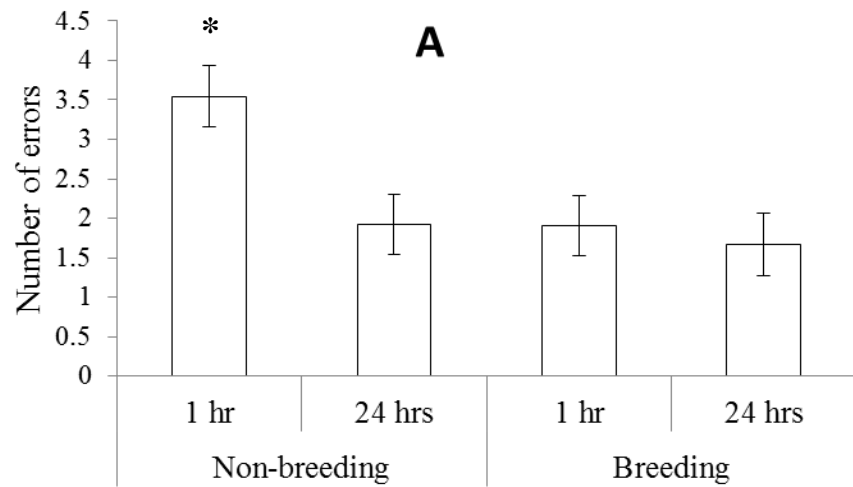


Figure 2.4

Table 2.1. Summary of statistical effects of sex, breeding condition (BC), and their interaction at the 24 h RI for each measure of performance during the matching phase of the search task. Significant factors are in bold.

Factors	<i>F</i>	df	<i>p-value</i>
Number of errors			
Sex	11.46	1,12	0.005
BC	0.33	1,11	0.58
Sex*BC	0.52	1,11	0.49
Search time			
Sex	1.48	1,12	0.25
BC	0.01	1,11	0.91
Sex*BC	0.00	1,11	0.95
Path tortuosity			
Sex	6.26	1,12	0.03
BC	0.02	1,11	0.89
Sex*BC	0.00	1,11	1.00

Search time

There was no effect of sex, breeding condition, or interaction between sex and breeding condition (Table 2.1, Figure 2.4C).

Path tortuosity

There was a significant effect of sex, with females having a less tortuous path than males (Table 2.1, Figure 2.2D). There was no effect of breeding condition or interaction between breeding condition and sex (Table 2.1, Figure 2.4D).

2.4 Discussion

Females made fewer spatial memory errors than males and took more direct paths to the rewarded cup (Table 2.1, Figures 2.4B, 2.4D). There was no effect of sex for search time (Figure 2.4C) indicating that females did not differ from males in motivation to search for the baited cup. This sex-specific effect may reflect an adaptation for brood parasitism because only females monitor host nests daily in the breeding season to appropriately time the parasitism event and maximize fitness (Rothstein et al. 1986, Gates and Evans 1998, Sealy 1992, White et al. 2009). We did not find a significant effect of breeding condition for any of the factors measured (Table 2.1 and Figure 2.4). However, because captivity may differentially affect the hippocampus I cannot preclude the existence of seasonal differences in free-living birds (Day et al. 2008). Regardless, where sex differences in spatial cognition are found in animals, it is usually males who have better spatial ability (Gaulin and Fitzgerald 1986). In contrast, I show superior female spatial ability in a system with sex-role-reversed use of space.

Although my spatial memory task did not specifically test memory for host nests, it likely tested for common underlying cognitive mechanisms which tap into the abilities that females use to re-visit host nests. Using food as a reward was necessary to ensure that both sexes would perform the task. Laboratory tests of spatial memory allow us to perform controlled tests of cognitive abilities that animals may use in their natural environment.

Female brown-headed cowbirds have a larger hippocampus than males, unlike related species that are not brood parasites (Sherry et al. 1993, Reboreda et al. 1996). Here, I show that this difference in brain morphology is associated with superior spatial memory in females as predicted from behavioural sex differences observed in the wild (Rothstein et al. 1986, Gates and Evans 1998). Female superiority in memory for spatial locations in brown-headed cowbirds suggests that spatial ability in this species has been adaptively modified for a brood-parasitic mode of reproduction.

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Chapter 3

3. Sex differences in spatial memory in brown-headed cowbirds: Males outperform females on a touchscreen task³

3.1 Introduction

Some animals show adaptive specialization of spatial ability (Sherry 2006, Smulders et al. 2010, Mettke-Hofmann 2014). Food-storing birds remember the locations of large numbers of scattered food caches. Spatial memory differs between food-storing and non-storing species (Balda et al. 1996, Shettleworth and Hampton 1998) and between populations of the same food-storing species that differ in their reliance on stored food (Pravosudov and Roth 2013). Adaptive specialization can also lead to sex differences in

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spatial ability. Polygynous male meadow voles (*Microtus pennsylvanicus*) have larger home ranges, better spatial memory, and a larger hippocampus than females, sex differences that are not found in monogamous species of *Microtus* where females and males have similar home ranges (Gaulin and Fitzgerald 1986, Gaulin and Fitzgerald 1989, Gaulin et al. 1990, Jacobs et al. 1990, Sherry et al. 1992). Similar sex differences in spatial memory occur in polygynous deer mice (*Peromyscus*) (Galea et al. 1996, Jašarević et al. 2012) and laboratory mice (Bettis and Jacobs 2013).

Sex differences in spatial ability are not, however, simply a matter of better or poorer performance by one sex or the other. Female and male laboratory rats and mice use different dissociable kinds of spatial information for orientation and navigation (Williams et al. 1990, Bettis and Jacobs 2013). Male mice and rats rely predominantly on geometric information (i.e., distant landmarks), whereas females use predominantly feature information (i.e., local landmarks; Williams et al. 1990, Rodríguez et al. 2011, Bettis and Jacobs 2013). Consistent with the use of different landmarks between the sexes, male mice, rats, and humans outperform females on spatial tasks requiring navigation through space or the visualization of three-dimensional or directional information, whereas females outperform males on smaller scale spatial tasks relating to object location (Williams et al. 1990, Silverman et al. 2000, Voyer et al. 2007, Rodríguez et al. 2011, Bettis and Jacobs 2013). Sex differences in the kind of information used for orientation are an organizational effect of exposure to gonadal steroids during development (Williams and Meck 1991).

Sex differences in spatial ability also occur in birds. Male hummingbirds (*Sephanooides sephanooides*) are better than females at remembering the location of high

quality nectar sources (González-Gómez et al. 2014) and female brood parasitic brown-headed cowbirds (*Molothrus ater*) are better than males at remembering the location of a previously baited food source (Chapter 2, Guigueno et al. 2014). Astié et al. (1998) found, in contrast, that female shiny cowbirds (*Molothrus bonariensis*) performed better than males when a colour cue indicated the location of food, but not when spatial location alone was associated with food. Sex differences in the use of spatial information, however, do not always occur in birds. Females of three species of hummingbirds (*Selasphorus rufus*, *Hylocharis leucotis*, *Eugenes fulgens*) have the same preference for spatial cues over featural cues that is found in males (Tello-Ramos et al. 2014) and the same is true of great tits (*Parus major*) (Hodgson and Healy 2005).

Some birds use spatial information differently depending on context. Noisy miners (*Manorina melanocephala*) revisit sites where they have *not* previously found food more often when searching for invertebrates than when searching for nectar (Sulikowski and Burke 2010). European greenfinches (*Carduelis chloris*) predominantly use colour cues to relocate a food site they have encountered only once previously, but switch to spatial cues after repeated encounters with food at the same site (Herborn et al. 2011).

Brown-headed cowbirds are obligate brood parasites and spatial ability likely plays a major role in the reproductive success of females. To successfully reproduce, females must find host nests and then re-visit these nests to assess the stage of completion of the host clutch, to lay their own eggs, and to remove host eggs (Norman and Robertson 1975, Rothstein et al. 1986, Gates and Evans 1998, White et al. 2009, Guigueno and Sealy 2011). Male brown-headed cowbirds do not search for or visit host nests. Females

might be expected to perform better than males on spatial memory tasks and indeed, female brown-headed cowbirds outperform males when searching for food hidden in baited cups within a large room (Chapter 2, Guigueno et al. 2014). The hippocampus is larger in female than in male brown-headed cowbirds (Sherry et al. 1993) and shiny cowbirds, another brood-parasitic species in which only females search for host nests (Reboreda et al. 1996). In screaming cowbirds (*Molothrus rufoaxillaris*), a related species of cowbird in which both sexes search for nests, females and males have a similar-sized hippocampus (Reboreda et al. 1996). The sex difference in favour of females in both navigational spatial ability and relative size of the hippocampus found in brown-headed cowbirds is thus the reverse of that usually found in mammals.

Female cowbirds only search for host nests during the breeding season. Changes in breeding condition influence hormone levels in both female and male brown-headed cowbirds (Chapter 2, Guigueno et al. 2014) and both testosterone and estradiol can influence acquisition and performance of spatial tasks (Oberlander et al. 2004, Hodgson et al. 2008, Bailey et al. 2013).

In the current study, I used operant conditioning to compare performance of female and male brown-headed cowbirds on a delayed-matching-to-sample (DMTS) touchscreen task. This task, unlike the task used by Guigueno et al. (2014) (Chapter 2), does not involve movement through a spatial environment but instead memory for a location in the immediate visual field. I investigated whether female cowbirds perform better than males in general or if superior female performance is associated only with tasks that resemble females' search for nests. I also compared performance between breeding and non-breeding conditions, inducing breeding condition by manipulation of

photoperiod. Finally, I compared memory for both spatial and colour cues on the touchscreen.

I predicted that if female cowbirds have better spatial ability than males in general, then females would perform better than males on the spatial but not on the colour DMTS touchscreen task. In contrast, spatial ability may depend on the nature of the task, like in rodents and humans. Because female cowbirds outperformed males on a previous spatial task that required movement through space (Chapter 2, Guigueno et al. 2014), I predicted that, if sex differences in cowbirds are task-dependent, performance on the spatial touchscreen task would be similar between the sexes or may favour males.

3.2 Methods

3.2.1 Subjects

I captured eight female and eight male cowbirds in April 2011 at the Queen's University Biological Station near Elgin, Ontario, Canada and transported them to the University of Western Ontario. I housed the birds indoors in individual cages and fed them *ad libitum* for two months until the beginning of training. During this two-month period, birds reached their captive free-feeding weight. Food consisted of a mix of seeds (50% Living World® Premium seed mix for budgies: 50% white millet), fruits, vegetables, a modified Bronx Zoo diet for omnivorous birds (eggs, carrots, molasses, brown rice, wheat germ, dog food, exotic gamebird starter, and turkey starter) and oyster shells.

3.2.2 Breeding condition manipulation and measurement

Photoperiod manipulation

Subjects were tested in non-breeding and breeding conditions (Table 3.1). Subjects were exposed to varying photophase lengths at different points throughout the study to induce photosensitive and photorefractory states (Dawson et al. 2000). Subjects in non-breeding condition had been housed on days with long photophases (16 h: 8 h light:dark [L:D] cycle) for several months, had moulted before testing began, and were thus in a non-reproductive photorefractory state (Dawson et al. 2000). The long photophase was maintained during the entire non-breeding testing period. I then switched birds to a short photophase (8 h L: 16 D) for 60 d to induce photosensitivity. During this period, I did not test birds and they were fed ad libitum. Next, I photostimulated birds with a moderately long photophase (14.5 h L: 9.5 h D) to bring them into breeding condition. Training to criterion began a week later. I confirmed birds were in non-breeding and breeding condition when photorefractory and photostimulated, respectively, by assaying blood samples from test subjects, noting the frequency of song in the housing room, and examining the gonads of other cowbirds housed in the same room.

Song recording and laparotomies

I recorded song frequency in the cowbird housing room from 10:00 to 13:00 EDT in the middle of testing in non-breeding condition, three times during breeding and once after testing. Sentinel cowbirds were laparotomized during non-breeding and breeding condition testing. Birds were anesthetised with isoflurane and a small incision was made on the left flank to allow gonad measurement.

Table 3.1. Sequence of testing conditions for all birds. Spatial and colour delayed-matching-to-sample (DMTS) tasks were performed in non-breeding condition (unshaded) and breeding condition (shaded) at varying retention intervals.

NON-BREEDING	
Spatial DMTS	Training to criterion Progressive retention intervals: 15 sessions Randomized retention intervals: 11 sessions + 3 testing sessions
Colour DMTS	Training to criterion Progressive retention intervals: 15 sessions Randomized retention intervals: 11 sessions + 3 testing sessions
BREEDING	
Spatial DMTS	Training to criterion Progressive retention intervals: 15 sessions Randomized retention intervals: 11 sessions + 3 testing sessions
Colour DMTS	Training to criterion Progressive retention intervals: 15 sessions Randomized retention intervals: 11 sessions + 3 testing sessions

Blood sampling

Touchscreen testing did not occur on blood sampling days, and subjects were not food deprived. For each breeding condition, two blood samples were taken during spatial testing and two more during colour testing, for a total of four samples from each subject per condition. Blood samples were collected within 30 min of entering the housing room. I punctured the brachial vein with a 26-gauge needle and collected approximately 300 μ L of blood into heparinized capillary tubes. Blood was kept cool and then centrifuged at 13 g for 10 min within 5 h of collection. The plasma was frozen in glass vials and stored in a freezer until hormone assay. For each breeding condition, two samples taken during spatial testing were pooled, as were the two samples taken during colour testing, for a total of two large samples per subject per breeding condition.

Androgen assay

Androgen concentration was assayed using a testosterone enzyme immunoassay (EIA; Cat. #1-2402, Salimetrics). Because the antibody in this kit cross-reacts with dihydrotestosterone and other androgens, I refer to measures as androgen levels. This kit has been validated in songbirds (Washburn et al. 2007). I diluted plasma samples five times and followed Newman et al. (2008) to validate the assay for cowbirds. I assayed a serial dilution of cowbird plasma and compared it to the standard curve using ANCOVA. A non-significant interaction term ($F_{1,9} = 0.42$, $p = 0.54$) indicated the slopes were similar ($R^2 = 0.84$) and that the assay was suitable for cowbirds. Inter-plate variation, based on a pooled cowbird plasma sample and low and high controls, was 9.41%. Intra-assay variation, based on variation between duplicates, was 9.43%. The sensitivity of my assay was 1 pg/mL (two standard deviations from the average value of zero on my four

standard curves). Samples below 1 pg/mL were assigned a value of 0.5 pg/mL for statistical analyses.

3.2.3 Touchscreen apparatus, training and software

Touchscreen chambers were 31 cm deep, 36 cm wide, and 34 cm high, and were housed in sound-attenuating booths (Eckel Noise Control Technologies, Morrisburg ON). Subjects pecked a computer monitor that made up one side of the chamber, and was surrounded by a CarrollTouch infrared touchscreen frame (Elo Touch Solutions[®], Rochester NY). Each monitor and touchscreen frame was connected to a computer that presented stimuli and recorded responses using Experimentor Software, a program that I helped create (McCarter 2012).

During training and testing for the touchscreen tasks, I food-restricted subjects to maintain them at 85% of their free-feeding weight, which was similar to their free-living body weight (i.e., body weight at capture from the wild). Naïve subjects were first placed in the operant chamber with the food hopper in the elevated position until the birds were feeding from the hopper. The hopper was then moved up and down randomly until the birds became habituated to the equipment noise and were feeding from the moving hopper. I then manually shaped the birds to peck a shape on the touchscreen to access the food hopper. I adhered a clear tape with seeds on the shape to encourage the birds to peck. It took between one to four 45-min hand-shaping sessions for all the birds to learn to peck a shape to bring the food hopper to the elevated position. Finally, I trained birds to peck progressively longer sequences of shapes until the full sequence for the task was reached.

3.2.4 Spatial and colour delayed-match-to-sample (DMTS) tasks

A fixation point was displayed until the bird initiated a trial by pecking it, after which this fixation point disappeared and a sample square (black outline – spatial task, coloured – colour task) was presented for a maximum of 90 seconds (Figure 3.1, Table 3.2). As soon as the bird pecked the sample square (usually less than 90 seconds), it disappeared and a retention interval (RI) of 5, 15, 30, 45, or 60 s with a blank white screen was displayed. After the RI, a second fixation point was presented for up to 5 s. Once pecked, the second fixation point disappeared and was replaced with a choice with three squares appearing for up to 10 s. If the sample square, the second fixation or a choice were not pecked within the allocated time, the trial was considered abandoned, a white screen was presented for 5 s and the first fixation point was displayed again to initiate a new trial.

All stimuli were presented within an 85 X 80 mm rectangle on the 340 X 270 mm screen. Fixation points were circles with 30 mm diameter and centred on the screen. The sample and choice (match and distractor stimuli) squares were 25 X 25 mm. Sample squares could appear anywhere around the fixation point, with a consistent distance of 30 mm between the middle of the fixation point and the middle of the sample square. The matching and distractor stimuli appeared 55 mm equidistant from each other rotated randomly around the fixation point and 35 or 65 mm from the sample square for the colour task (see section below for more details). Apart from this constraint, matching and distractor squares could appear at random anywhere around the fixation point except on the spatial task in which one stimulus always occupied the same location as the sample stimulus. The match and distractor stimuli could be no closer to each other than to the

Figure 3.1. Spatial (top) and colour (bottom) delayed-matching-to-sample (DMTS) touchscreen tasks. Every trial began with a fixation point (first image from left), followed by a sample square (second image from left). The bird had to remember either the location (spatial DMTS) or the colour (colour DMTS) of the sample square. After pecking the sample square, it disappeared and a retention interval (RI) of 5, 15, 30, 45 or 60 s with a blank white screen was displayed. After the RI, a second fixation point (third image from left) was displayed. The bird had to peck the second fixation point to have a choice of three squares displayed (last image). The correct square was either in the same location (spatial DMTS) or the same colour (colour DMTS) as the sample square. A correct choice resulted in 5 s of food access with a blank white screen and an incorrect choice resulted in 5 s without food access with a blank black screen.

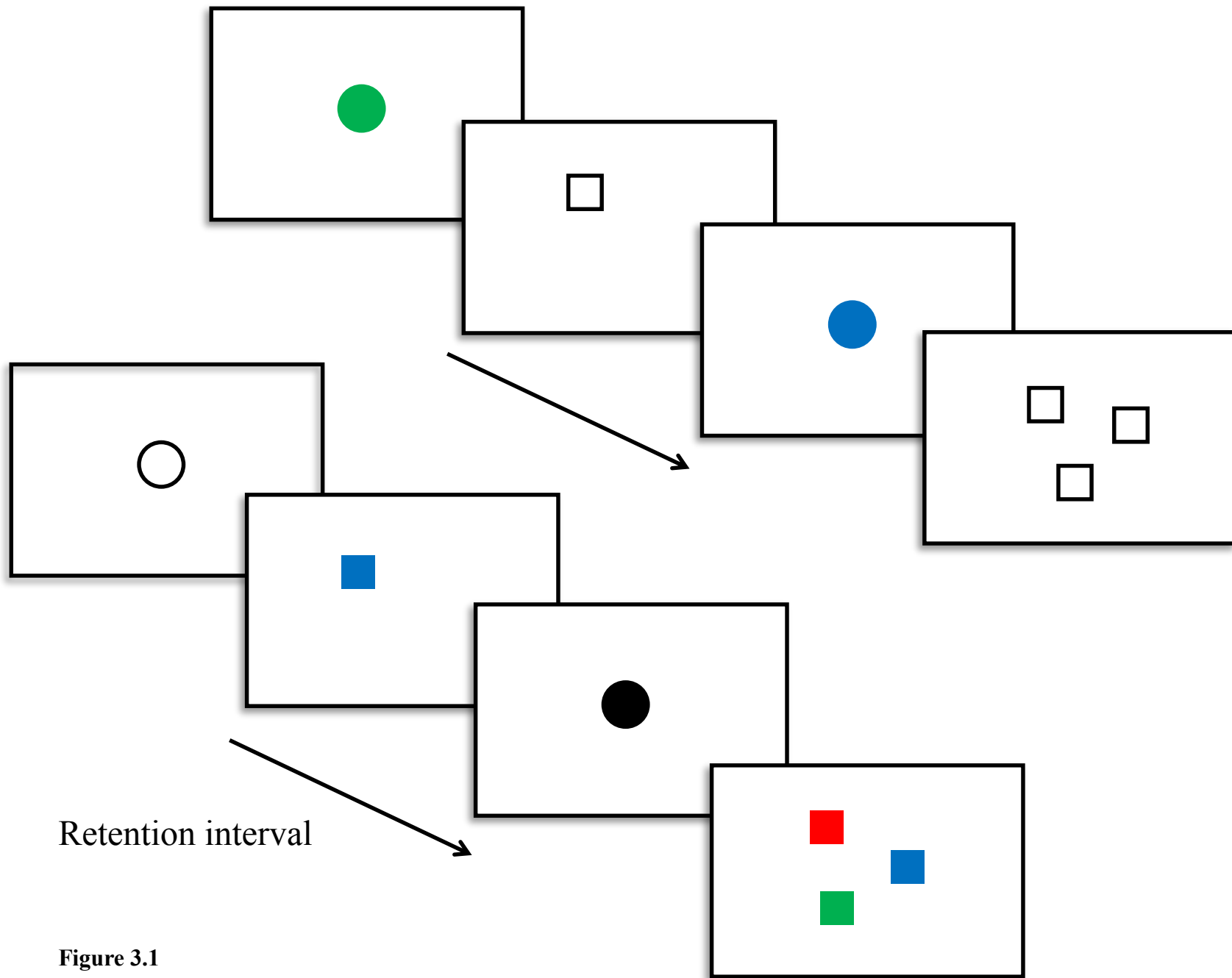


Figure 3.1

Table 3.2. HTML codes for the colours used in the colour DMTS task.

Colour name	HTML code
Green	#55AA2B
Red	#FF0000
Blue	#0000FF
Fuchsia	#FF00FF
Orange	#CC6600
Purple	#800080
Olive	#808000
Dark blue green	#4C7D7E
Brown	#A52A2A
Gold	#EAC117

comparison stimuli. Colour stimuli for the colour DMTS task could have one of 10 colours. Colours were drawn from HTML codes (Table 3.2).

I tested subjects first with a spatial DMTS task, then a colour DMTS task in non-breeding and breeding conditions (Table 3.1). A correct choice was rewarded with 5 s of food access and an incorrect choice was followed by a black screen that darkened the chamber for 5 s. Each subject was tested in 1.5 h daily sessions during which subjects completed as many trials as possible (from 20 to over 200 trials, depending on RI length) to generate the most accurate measure of performance (proportion correct). The illumination level of the white screen on which stimuli were presented was 116.3 lux and the illumination level of the black screen was 2.2 lux measured at the position of the bird in the chamber.

Subjects were trained on a 0-s RI task and had to complete at least 5 sessions and reach a criterion of two sessions with $\leq 10\%$ variation and accuracy of at least 10% above random performance (Training to criterion phase; see Table 3.1). Once criterion at a 0 s RI was reached, the RI was increased progressively every fourth session for 15 sessions (Progressive RI phase; see Table 3.1) with a sequence of RIs as follows: 5, 15, 30, 45 and 60 s. The Progressive RI phase was followed by 14 sessions in which the same RI (except 5 s) occurred in a random sequence within each session (Randomized RI phase). The Progressive RI phase and the first 11 sessions in the Randomized RI phase were considered training sessions prior to the subjects reaching their peak performance before subsequent testing for each task type (spatial and colour) and for each breeding condition (non-breeding and breeding) as performance significantly improved over these sessions (Table 3.1). Analyses of the Progressive RI phase were not the focus of this chapter, but

are included in the results. Only the last three sessions in the Randomized RI phase were used as testing sessions to assess the subjects' peak performance described in the sections "DMTS tasks" and "Comparison between spatial and colour tasks" below. In the section "Transition between the spatial and colour tasks", I used data from the absolute first three sessions on the colour task (Training to criterion phase in Table 3.1) and the first three sessions in the Progressive RI phase of the colour task, both in non-breeding condition (described in more detail below; Table 3.1).

Each subject thus had extensive training by the onset of the testing sessions, in order to minimize the effects of order of testing (spatial before colour, and non-breeding before breeding; Table 3.1). One female subject did not reach criterion for the colour task in non-breeding and breeding conditions, therefore I only include her data for the spatial task in non-breeding and in breeding conditions. Removing this female from the analyses did not change the main conclusions of the study. For spatial testing in breeding condition, data were absent for two out of the 8 males because one male died and the other fell ill and could not be used for testing during that period. For colour testing in breeding condition, data were missing from the male that previously died and a female that died during that period. Final sample sizes were as follows: non-breeding spatial: 8 females, 8 males; non-breeding colour: 7 females, 8 males; breeding spatial: 8 females, 6 males; breeding colour: 6 females, 7 males. In addition to analyzing performance of females and males for each task type (spatial and colour), I analyzed differences in performance between the spatial and colour tasks for each sex.

3.2.5 Transition between spatial and colour DMTS tasks and breeding conditions

I determined whether the spatial location of the rewarded square affected the subjects' choice on the colour test. The squares used in the spatial DMTS task were always white with a black outline and so cowbirds could not use colour to solve the spatial DMTS task. Cowbirds could have persisted in using spatial location of the sample during the colour DMTS task, however, so I ensured the cowbirds were sufficiently trained on the colour DMTS task before colour testing began. To determine whether location affected the cowbirds' choice during colour testing, I recorded the distance between the location of the sample square presented immediately after fixation point one and the location of the correct matching square during the choice phase. I then determined whether distance influenced the probability of making a correct choice. The correct square was never presented in the same location as the sample square for the colour task. The distance between the sample and correct squares could either be "near" or "far" because the three choice squares were always equidistant from each other and centered around the fixation point, but shifted either 60° ("near") or 120° ("far") from the original location of the sample square (Figure 3.1). With this information, it was possible to measure the influence of distance on the probability of making a correct choice.

To ensure that performance was not affected by the order of testing of non-breeding and breeding conditions, birds repeated the entire sequence of training to criterion followed by Progressive Retention Interval (RI) training before Randomized RI testing began for both spatial and colour tasks (see Table 3.1).

3.2.6 Statistical analyses

I analyzed my data with linear mixed models (PROC MIXED in SAS 9.3; SAS Institute, Cary NC) because the dependent variables were continuous, repeated measures, and data were missing at random due to the loss of some subjects during the experiment (see above). I used a compound symmetry covariance structure because the model AIC values were lower than with the default variance components covariance structure (see also Jenrich and Schluchter 1986). Data were appropriately transformed for analyses (see details below) to produce normally distributed residuals. However, to facilitate interpretation, data in figures are presented as untransformed means \pm SE, except for significant interactions for the spatial and colour DMTS tasks, which are presented as transformed least squares means \pm SE. I analyzed all two-way interactions as factors in the models. Significant effects were further analyzed using Fisher's LSD post-hoc tests.

Androgen concentrations

I used a linear mixed model with sex, breeding condition, and task type as fixed factors and subject as a random variable to analyze androgen concentrations. Data were log-transformed for analyses to produce normally-distributed residuals.

DMTS tasks

I used a linear mixed model for each task type (spatial and colour) with RI (15 s, 30 s, 45 s, and 60 s), breeding condition (non-breeding and breeding), and sex (female and male) as fixed factors and subject as a random variable to analyze the proportion of correct responses, which were arcsine square root transformed for the analyses to produce normally-distributed residuals (Sokal and Rohlf 1995). The dependent variable was peak performance (i.e., the last three sessions) from the Randomized RI phase (Table 3.1).

Comparison between spatial and colour tasks

I used a linear mixed model with task type (spatial and colour) and sex (female and male) as fixed factors and subject as a random variable to analyze the proportion of correct responses in the testing sessions, which were arcsine square root transformed for the analyses to produce normally-distributed residuals. The dependent variable was peak performance (i.e., the last three sessions) from the Randomized RI phase for the spatial and colour tasks (Table 3.1).

Transition between the spatial and colour tasks

Birds performed the spatial task before the colour task first in non-breeding condition and then again in breeding condition (Table 3.1). To determine whether birds attempted to use a spatial matching strategy to solve the colour matching task, I measured the distance between the sample and the correct square for each trial during the first three training to criterion sessions on the colour task (RI = 0 s) and during the first three sessions in the Progressive RI phase (RI = 5 s) of the colour task in non-breeding condition. For all birds, this was the first transition from the spatial to the colour task (Table 3.1). I used linear mixed models for the first three sessions during the initial

training and Progressive RI phases with distance between the sample and chosen stimuli and sex as fixed factors and subject as a random variable to analyze the proportion of correct responses. The proportion of correct responses were log arcsine square root transformed for the initial training phase and arcsine square root transformed for the Progressive RI phase to produce normally-distributed residuals.

3.3 Results

3.3.1 Confirmation of breeding condition

Song frequency

Song frequency in non-breeding condition (147 songs per hour) was about a tenth of the frequency recorded in breeding condition (1224, 1098, and 969 songs per hour). After the DMTS testing was completed and the birds moulted, song frequency decreased to a non-breeding level (72 songs per hour).

Laparotomies

I performed laparotomies on sentinel birds (i.e., birds not used in the DMTS tasks) during non-breeding and breeding conditions to record changes in gonadal development. I measured the testes of three males twice in non-breeding condition (range 1.5-2 mm) and two males twice in breeding condition (range 3-4 mm), confirming testicular development. The ovaries of three females examined twice in non-breeding condition were granular and showed no follicle hierarchy. I did not perform laparotomies on females in breeding condition because a change in gonadal development in females is generally only seen a few days before egg-laying (SMS, pers. obs.). Near the end of

testing in breeding condition, however, three females in the colony laid eggs. One bird laid a single egg, another laid three eggs and one laid one egg per day for 14 consecutive days, confirming that females underwent gonadal development.

Androgen levels

There was a significant effect of breeding condition, with the highest circulating levels of androgens in breeding condition ($F_{1,14} = 120.63, p < 0.0001$), whereas there was no main effect of sex ($F_{1,14} = 2.82, p = 0.12$), or task type ($F_{1,14} = 1.08, p = 0.32$; Figure 3.2). There was a significant sex by breeding condition interaction ($F_{1,14} = 22.61, p = 0.0003$), with a greater effect of breeding condition in males ($t_{14} = 11.02, p < 0.0001$) than in females ($t_{14} = 4.45, p = 0.0005$; Figure 3.2). There was also a significant task type by breeding condition interaction ($F_{1,14} = 5.95, p = 0.03$), with a greater change in androgen concentration between breeding conditions for the spatial task ($t_{13} = 9.59, p < 0.0001$) than for the colour task ($t_{13} = 5.98, p < 0.0001$; Figure 3.2). There was no significant interaction between sex and task type ($F_{1,14} < 0.01, p = 0.99$).

3.3.2 Acquisition: Progressive RI

Spatial memory

Performance decreased as RI increased ($F_{4,52} = 67.69, p < 0.0001$). Sex, breeding condition, and all interactions were non-significant (Table 3.3, Figure 3.3).

Colour memory

Subjects performing better in breeding condition than in non-breeding condition ($F_{1,12} = 47.11, p < 0.0001$). Performance decreased as RI increased ($F_{4,52} = 22.33, p < 0.0001$). Sex and all interactions were non-significant (Table 3.4, Figure 3.3).

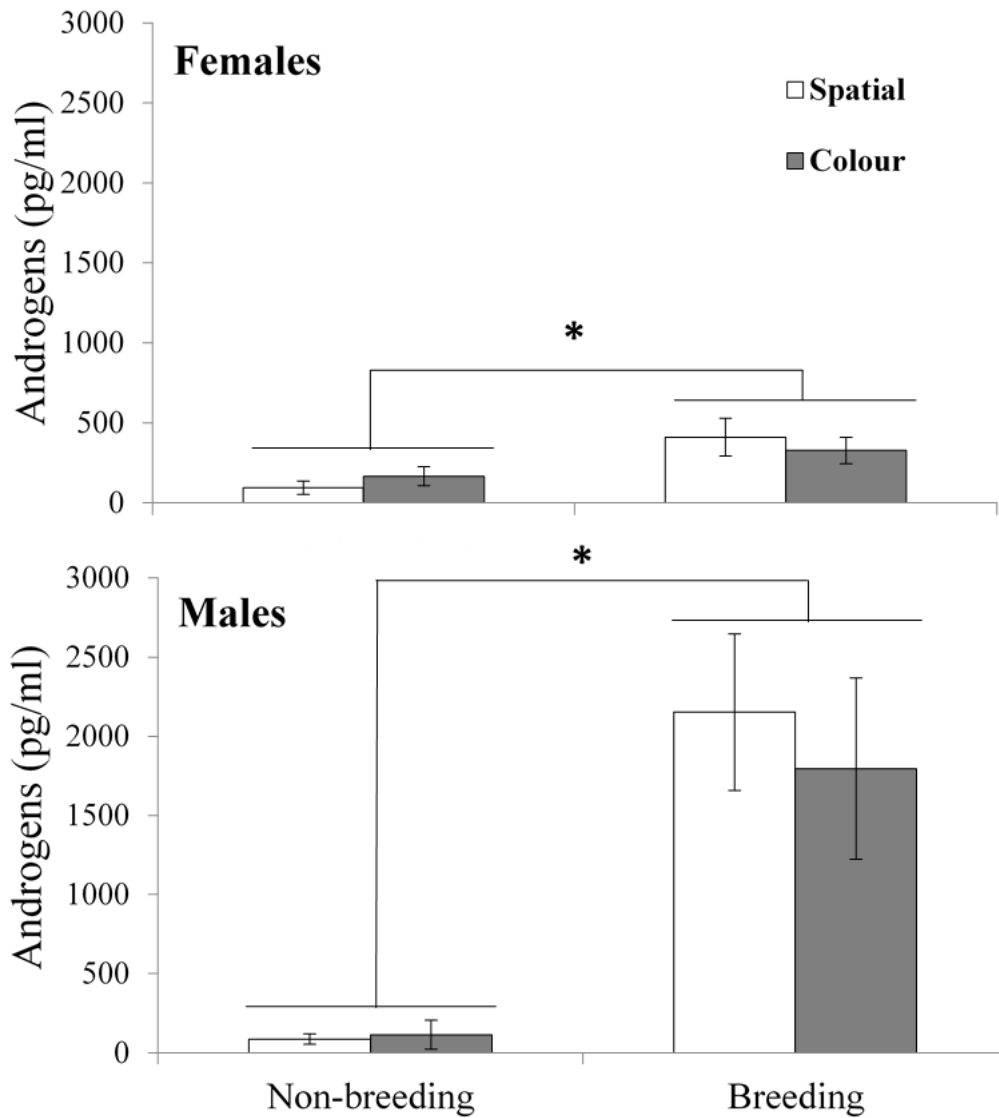
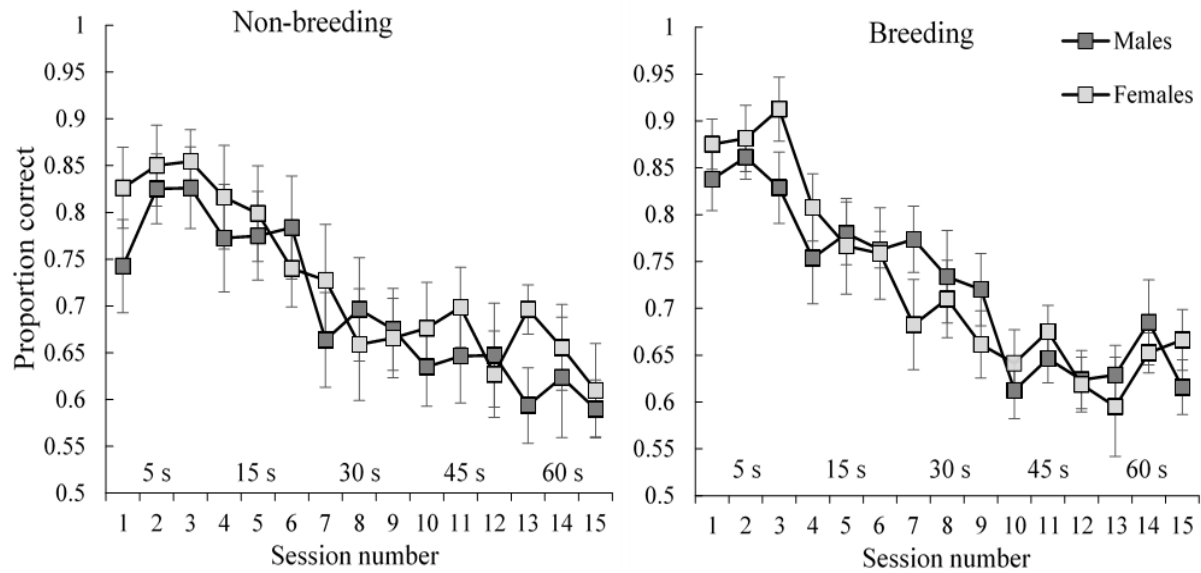


Figure 3.2. Plasma androgen concentrations in female and male brown-headed cowbirds between breeding conditions and performance on spatial and colour tasks. Means are presented with \pm SE. Asterisks indicate $p \leq 0.05$.

Table 3.3. Summary of statistical effects of sex, breeding condition (BC), retention interval (RI) and their interactions during the 15 practice sessions from the Progressive RI phase and the 3 test sessions from the Random RIs phase for the spatial delayed-matching-to-sample task. Significant effects are in bold.

Factors	<i>F</i>	d.f.	<i>p</i>
Spatial			
Progressive RI – 15 practice sessions			
Sex	0.31	1,14	0.59
BC	1.48	1,14	0.24
RI	67.69	4,56	<0.0001
Sex*BC	1.86	1,14	0.19
Sex*RI	1.51	4,56	0.21
BC*RI	1.61	4,60	0.18
Randomized RI – 3 test sessions			
Sex	1.77	1,14	0.20
BC	0.49	1,12	0.50
RI	0.70	3,42	0.56
Sex*BC	11.66	1,12	0.005
Sex*RI	4.15	3,42	0.01
BC*RI	0.02	3,39	1.00

Spatial



Colour

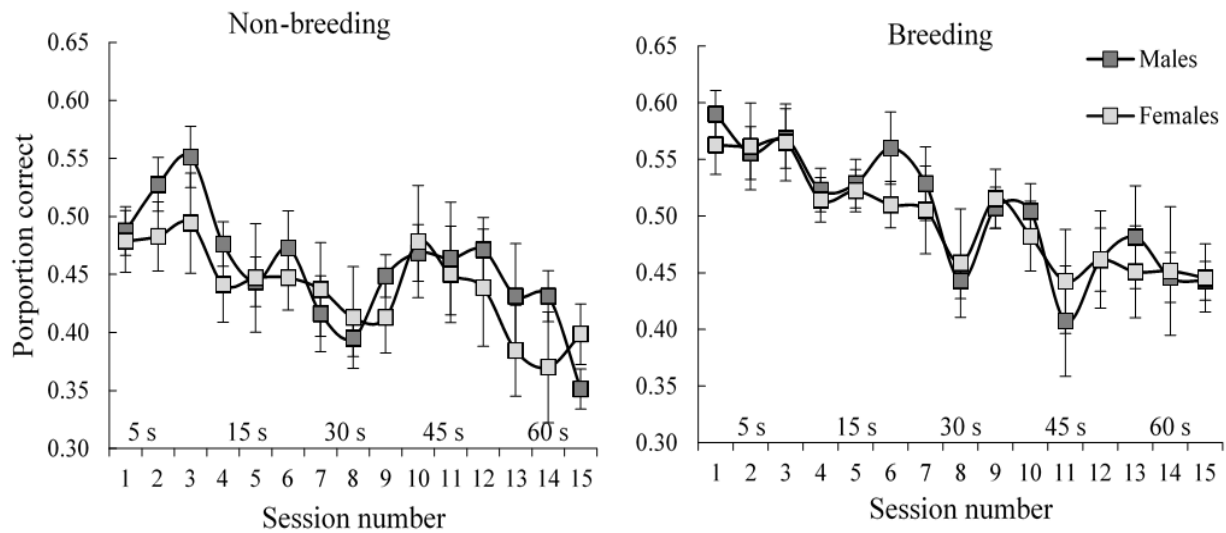


Figure 3.3. Mean performance \pm SEM on the Progressive RI spatial (top) and colour (bottom) delayed-matching-to-sample tasks in non-breeding and breeding conditions. The retention interval was progressively increased after three sessions at a given retention interval.

Table 3.4. Summary of statistical effects of sex, breeding condition (BC), retention interval (RI) and their interactions during the 15 practice sessions from the Progressive RI phase and the 3 test sessions from the Random RIs phase for the colour delayed-matching-to-sample task. Significant effects are in bold.

Factors	<i>F</i>	d.f.	<i>p</i>
Colour			
Progressive RI – 15 practice sessions			
Sex	0.67	1,13	0.43
BC	47.11	1,12	<0.0001
RI	22.33	4,52	<0.0001
Sex*BC	0.21	1,12	0.65
Sex*RI	0.57	4,52	0.68
BC*RI	1.79	4,49	0.15
Randomized RI – 3 test sessions			
Sex	0.00	1,13	0.98
BC	10.91	1,11	0.007
RI	6.01	3,39	0.002
Sex*BC	13.68	1,11	0.004
Sex*RI	0.30	3,39	0.82
BC*RI	1.86	3,36	0.15

3.3.3 Testing: Randomized RI

Spatial memory

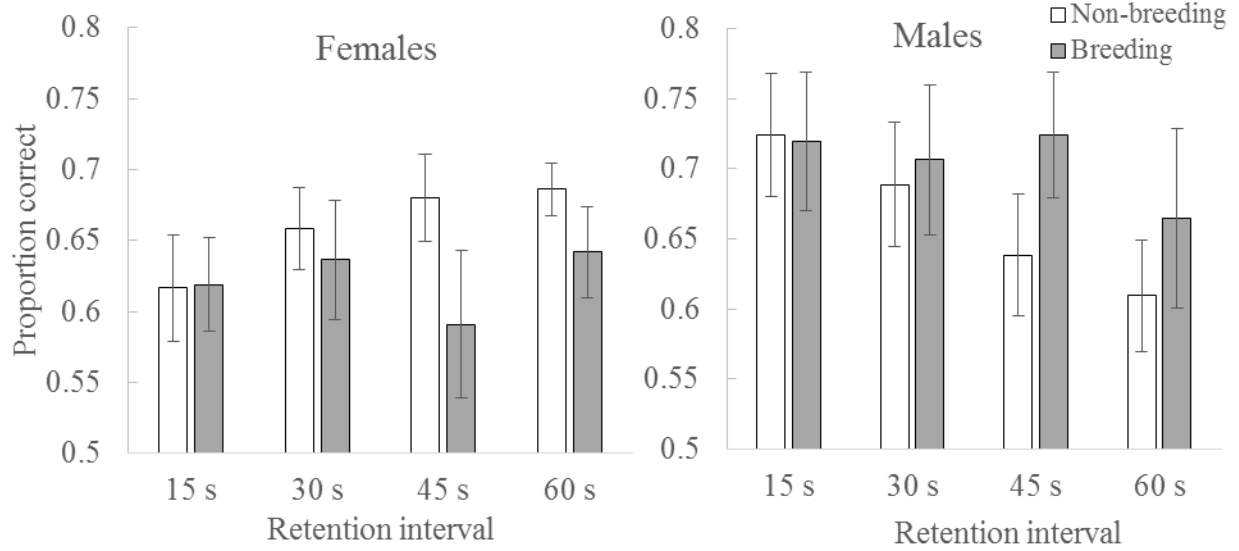
There were no main effects of sex, breeding condition, or RI and no significant breeding condition by RI interaction on performance (Table 3.3, Figure 3.4A). There was, however, a significant sex by breeding condition interaction ($F_{1,12} = 11.71, p = 0.005$; Figure 3.4B). Breeding males performed better than breeding females ($t_{12} = 2.42, p = 0.03$; Figure 3.4B). Males performed better in breeding condition than in non-breeding condition ($t_{12} = 2.70, p = 0.02$) whereas females performed nearly significantly better in non-breeding condition than in breeding condition ($t_{12} = 2.11, p = 0.06$; Figure 3.4B). There was also a significant sex by RI interaction ($F_{3,42} = 4.06, p = 0.01$), with males performing better than females at the 15 s RI ($t_{42} = 2.57, p = 0.01$; Figure 3.4C). Males performed more poorly at the 60s RI compared to the 15 s RI ($t_{42} = 3.19, p = 0.003$), whereas females' performance remained stable ($t_{42} = 1.67, p = 0.10$; Figure 3.4C).

Colour memory

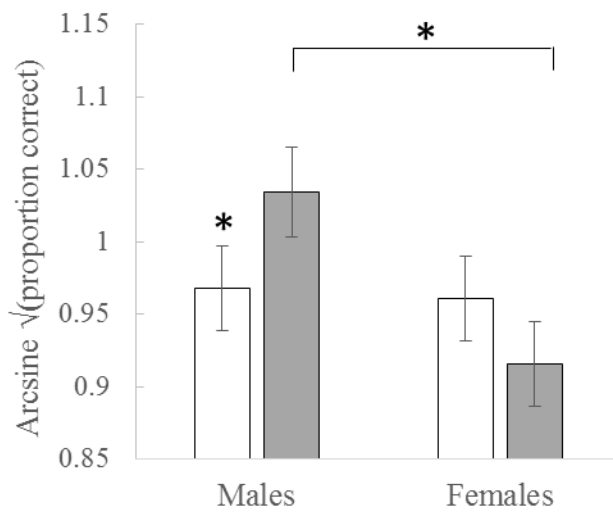
There was a significant main effect of breeding condition ($F_{1,11} = 8.60, p = 0.01$; Figure 3.5A). However, a significant sex by breeding condition interaction ($F_{1,11} = 12.41, p = 0.005$) indicated that only females performed better in breeding condition than in non-breeding condition ($t_{11} = 4.40, p = 0.001$), with males' performance showing no difference between breeding conditions ($t_{11} = 0.44, p = 0.67$; Figure 3.5A). There was also a significant main effect of RI, with performance decreasing as RI increased. There was also a significant main effect of RI, with performance decreasing as RI increased ($F_{3,39} = 5.52, p = 0.003$; Figure 3.5A). There was no effect of sex and all other interactions were not significant (Table 3.3).

Figure 3.4. A) Peak performance on the spatial delayed-matching-to-sample touchscreen task. Performance was calculated from the last three sessions of the with randomized retention intervals phase. Means of raw data are presented with \pm SE. Proportion correct expected by chance equals 0.33. Two males were only tested in non-breeding condition and elevated raw means in this condition more heavily than the linear mixed models, for which least squares means of significant interactions are presented in B and C. B) Summary by sex and breeding condition of data shown in A), with data arcsine square root transformed with least squares means \pm SE. Breeding males were significantly better than non-breeding males, but there was no difference between non-breeding and breeding females. In addition, breeding males performed significantly better than breeding females. C) Summary of data shown in A) by retention interval (RI), with data arcsine square root transformed with least squares means \pm SE. Males performed significantly better than females at the 15 s RI. Males performed significantly worse at the 60 s RI than at the 15s RI. Females are in light grey and males are in dark grey. Asterisks indicate $p \leq 0.05$.

A



B



C

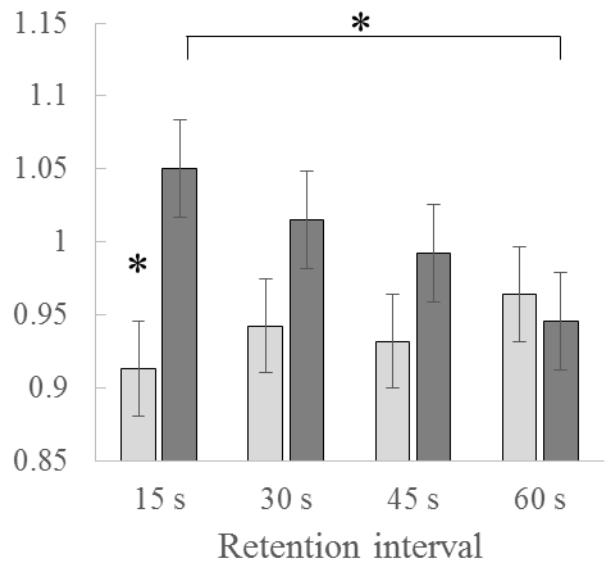
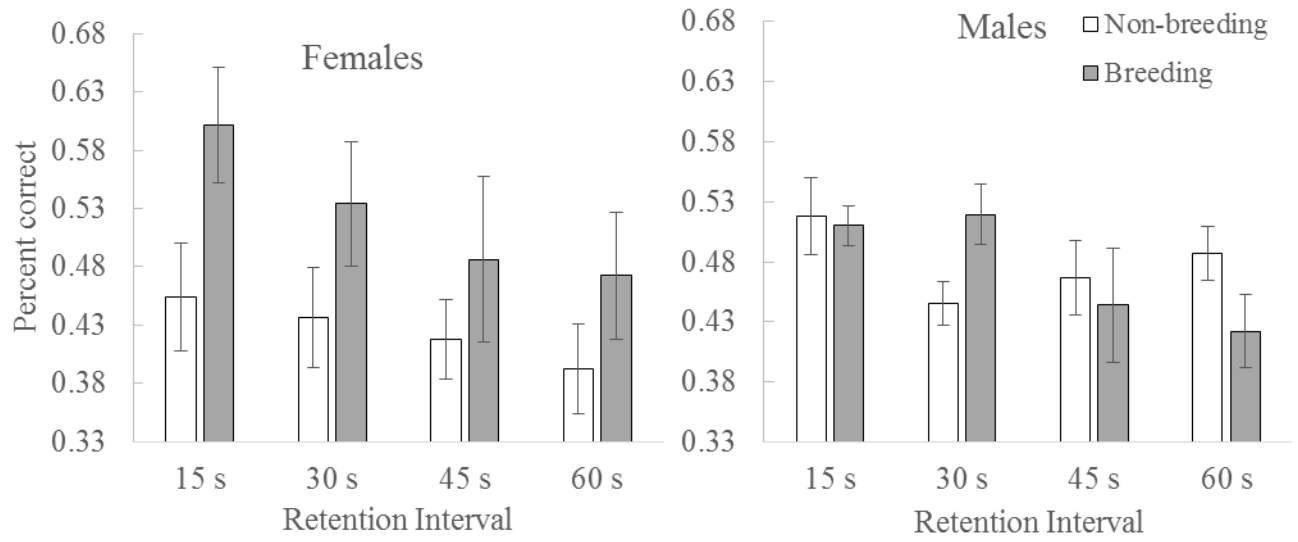


Figure 3.4

Figure 3.5. A) Peak performance on the colour delayed-matching-to-sample touchscreen task. Performance was calculated from the last three sessions of the randomized retention intervals phase. Means of raw data are presented with \pm SE. Proportion correct expected by chance equals 0.33. One female and one male were only tested in non-breeding condition and these missing points were corrected for in the linear mixed model, for which the only significant interaction is shown in B. B) Summary of the data shown in A) by sex and breeding condition, with data arcsine square root transformed with least squares means \pm SE. Females performed significantly better in breeding than in non-breeding condition, with no effect of breeding condition for males. Asterisks indicate $p \leq 0.05$.

A



B

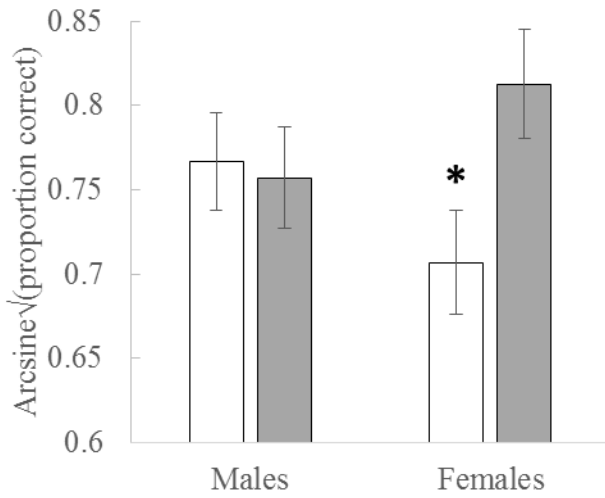


Figure 3.5

3.3.4 Comparison between spatial and colour tasks

Performance differed significantly between task types ($F_{1,13} = 310.20$, $p < 0.0001$), with cowbirds performing better on the spatial task than on the colour task (Figure 3.6). There was no significant main effect of sex ($F_{1,14} = 1.22$, $p = 0.29$) and no significant sex by task interaction ($F_{1,13} = 1.10$, $p = 0.31$; Figure 3.6).

3.3.5 Transition between spatial and colour tasks

During the initial transition from the spatial to the colour task (i.e., in non-breeding condition), birds were more likely to respond correctly when the correct colour match was near the spatial location of the sample than when it was further away ($F_{1,13} = 43.96$, $p < 0.0001$) (Figure 3.7A). There was no main effect of sex ($F_{1,13} = 0.60$, $p = 0.45$; Figure 3.7A), but there was a nearly significant interaction between sex and distance ($F_{1,13} = 4.42$, $p = 0.06$), with distance affecting males ($t_{13} = 6.32$, $p < 0.0001$) more than females ($t_{13} = 3.13$, $p = 0.008$; Figure 3.7A).

When birds were trained on the Progressive RI in non-breeding condition, following two weeks of colour training and the birds had reached criterion for the colour task, there was no longer an effect of distance on performance ($F_{1,13} = 0.28$, $p = 0.61$; Figure 3.7B). There was no main effect of sex ($F_{1,13} = 0.90$, $p = 0.36$) and no significant sex by distance interaction ($F_{1,13} = 0.36$, $p = 0.56$; Figure 3.7B). Thus, birds initially used a spatial strategy to solve the colour task, but by the beginning of Progressive RI training on the colour task, they no longer did so.

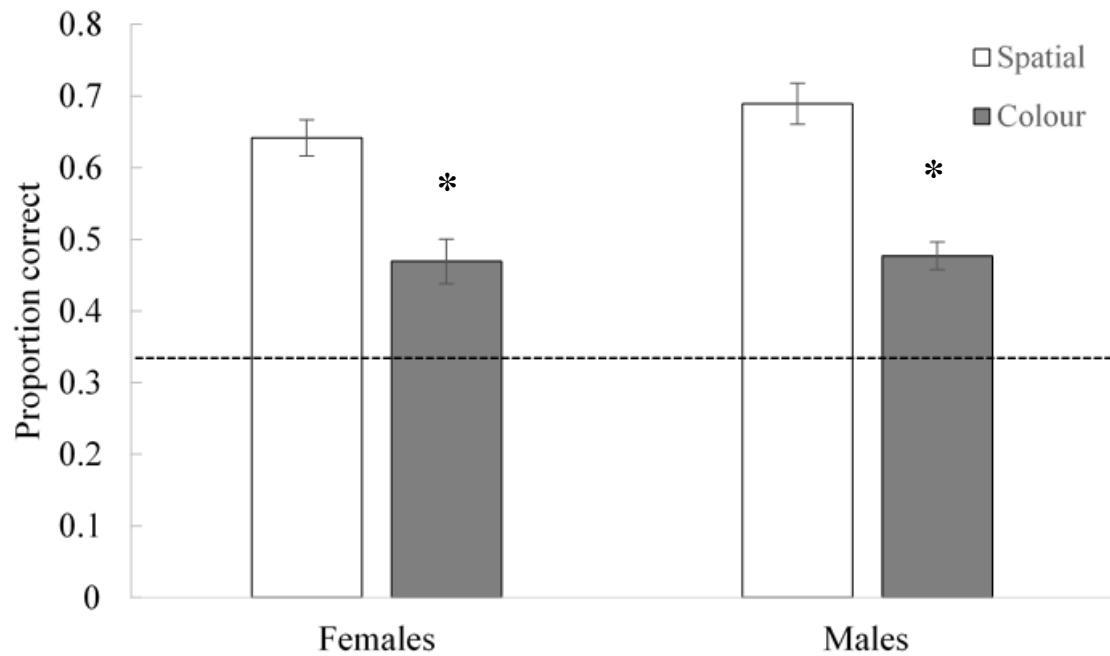


Figure 3.6. Comparison of performance between the spatial and colour tasks during the testing sessions (see Table 3.1). Female and male cowbirds performed better on the spatial task than on the colour task. Performance of both males and females was significantly better than chance, indicated by the dashed line. Means are presented with \pm SE. Asterisks indicate $p \leq 0.05$.

Figure 3.7. Transition from spatial to colour delayed-matching-to-sample touchscreen tasks. The figure shows the proportion of trials correct in the colour task when the correct match was near or far from the spatial location of the sample. A) In the first three sessions on the colour task (retention interval [RI] = 0 s), both males and females were more likely to make the correct colour choice when the matching stimulus was near the location the sample had occupied. During this phase of training, there was only one distractor presented in the choice phase, therefore random performance was 0.5 (indicated by dashed line). B) After two weeks of testing, when the Progressive RI testing phase began (RI = 5 s), the location of the matching colour stimulus had no effect on performance. During this testing phase, two distractors were presented in the choice phase therefore random performance was 0.33 (indicated by dashed line). Means are presented with \pm SE. Asterisks indicate $p \leq 0.05$ for the spatial versus colour comparison.

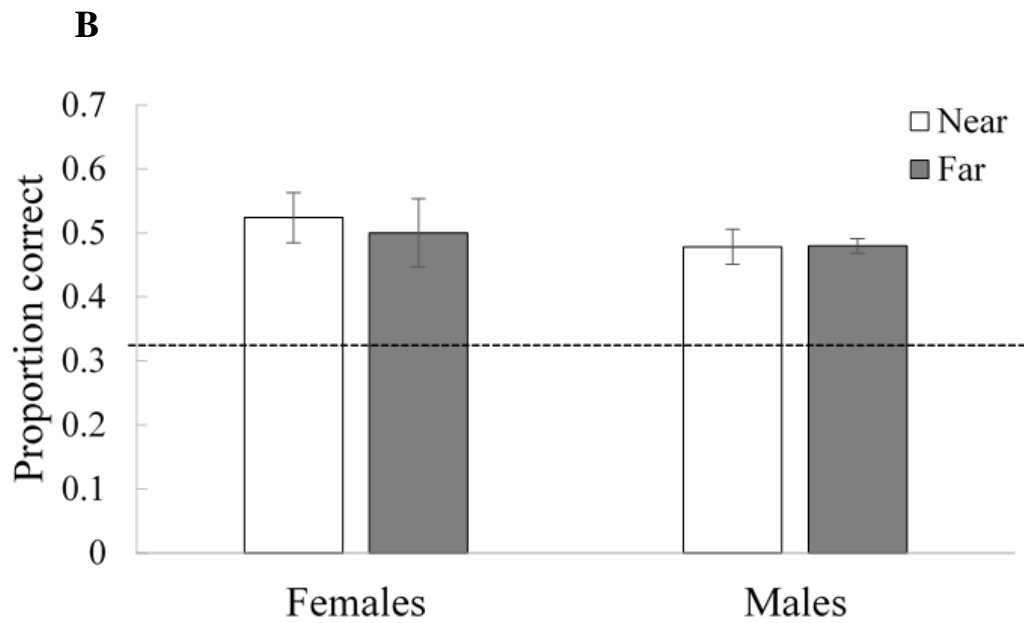
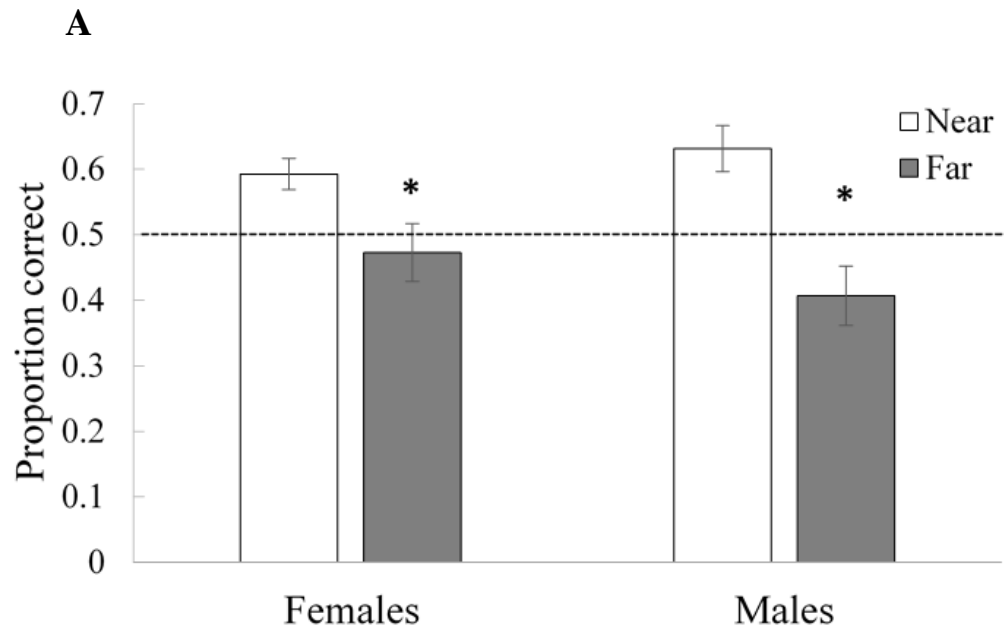


Figure 3.7

3.4 Discussion

Male cowbirds were more accurate than females on the spatial touchscreen task in two ways: first, males performed better than females at a short RI (15 s) and second, breeding males performed better than breeding females (Figure 3.4). On the colour task, females performed better in breeding than in non-breeding condition, whereas males' performance remained stable between breeding and non-breeding conditions (Figure 3.5). Finally, females and males both performed better on the spatial task than on the colour task (Figure 3.6).

Order effects could have potentially influenced differences in performance between tasks and between breeding conditions, because cowbirds were tested on the spatial task before the colour task and in non-breeding condition before breeding condition (Table 3.1). However, I found that order effects were negligible for task order because spatial location did not influence performance during the Progressive RI phase of the colour task, several sessions before colour testing began (Figure 3.7). This lack of order effects was likely due to extensive practice on the colour task before testing began (Table 3.1). In a similar fashion, birds were trained once again to asymptotic performance in breeding condition before the onset of the Progressive RI phase so that birds began training with increasing RI while at their peak performance at a 0 s RI (Table 3.1). I would expect birds of both sexes to perform better in breeding condition on both tasks if order effects were the main factors influencing differences between breeding conditions. However, males' performance remained stable between breeding conditions on the colour task (Figure 3.5) and females performed nearly significantly better in non-breeding condition than in breeding condition on the spatial task (Figure 3.4). I cannot fully

disentangle breeding condition versus non-breeding condition from the passage of time and any other factors potentially correlated with time. These other explanations are possible, but I find them unlikely.

3.4.1 Spatial memory

In contrast to the results of the current study, female cowbirds had more accurate spatial memory than males when navigating through a room to find baited food cups (Chapter 2, Guigueno et al. 2014). Although the task in that prior study and the touchscreen task described here were both DMTS tasks assessing spatial memory, the two tasks differed in spatial scale (180 cm X 180 cm versus 8.5 X 8 cm) and retention interval (24 h versus 5-60 s). Furthermore, the tasks differed in the response required of the birds; approaching and feeding from a cup versus pecking a symbol on a screen. Thus, whether or not a sex difference in spatial memory in cowbirds is observed depends on the spatial task.

Sex differences in spatial ability can be task-dependent. Males, especially in mammals, perform better than females on a variety of spatial tasks and a consistent feature of their enhanced performance is the use of spatial cues (geometric properties of the environment, distance, and direction) and feature cues while females prefer to use only feature cues (Gaulin et al. 1986, Gaulin et al. 1989, Silverman et al. 2000, Postma et al. 2004, Jozet-Alvez et al. 2008). However, recent work in birds showed that preference for spatial cues is not restricted to males but depends on the value of a cue to the solution of the task (Hodgson and Healy 2005, Tello-Ramos et al. 2014). Female cowbirds may have had a preference for spatial cues over feature cues on the task in (Guigueno et al.

2014), but this preference was reduced or absent in the current task. Selection may have led to flexibility in cue use in cowbirds rather than consistently superior spatial ability by one sex or the other (Hodgson and Healy 2005, Tello-Ramos et al., 2014). Finally, females performed equally well, and above chance, across retention intervals while male performance declined significantly with retention interval (Figure 3.4). Females may be relatively unaffected in general by retention interval. In the wild, females probably remember the locations of potential host nests for at least 24 h and females outperformed males at the 24 h retention interval tested in Chapter 2 (Guigueno et al. 2014).

Why did male cowbirds outperform females in breeding condition? From a life-history perspective, there could be a trade-off associated with specialization in a particular form of memory. Enhanced cognitive function has metabolic and life-history costs (Hasenlaub et al. 2010, Burns et al. 2011, Cole et al. 2012, Healy 2012). Improved ability in one type of spatial memory may come at the cost of another type of spatial memory. Female cowbirds did better on an allocentric task in which they moved through their environment and this ability may help females find and relocate host nests (Chapter 2, Guigueno et al. 2014). Enhanced performance by female cowbirds in allocentric spatial tasks may come at a cost in the performance of egocentric tasks such as my spatial touchscreen task in the current study. It is also possible that there is differential selection on males – for unknown reasons – for the ability to remember the location of objects in a spatial array in their immediate visual field. Finally, it is possible there is some functional incompatibility (Sherry and Schacter 1987) – again for reasons that are not known – between different kinds of spatial ability involved in orientation and navigation and remembering the location of objects in an array. In humans, men outperform women

on several measures of wayfinding through a wooded area or a large indoor environment (Silverman et al. 2000, Postma et al. 2004) but women outperform men on stationary object location memory tasks (Voyer et al. 2007, Hampson 2008). In cowbirds, observed sex differences on these different kinds of spatial memory are reversed relative to humans.

A proximate explanation for superior male performance in breeding condition could be their sex steroid hormone levels. Although androgen concentrations increased in both sexes between breeding conditions, males' concentration increased a great deal more between non-breeding and breeding conditions (Figure 3.2). Male deer mice show better acquisition of spatial maze performance than females in breeding condition only, when their testosterone levels are highest (Galea et al. 1996). In a task similar to mine, exogenous androgens improved performance on a delayed-non-matching-to-sample touchscreen task in great tits (Hodgson et al. 2008). In situ hybridization shows expression of androgen and estrogen receptor genes in the hippocampus of tits and testosterone and estrogen could influence spatial memory by binding to these receptors (Hodgson et al. 2008). The songbird hippocampus expresses high levels of aromatase, resulting in high levels of local estrogen synthesis from testosterone and enhanced spatial memory acquisition and performance (Saldanha et al. 2004, Oberlander et al 2004, Bailey et al. 2013). Enhanced performance on the DMTS spatial task by male cowbirds could thus have been caused by elevated testosterone levels.

3.4.2 Colour memory

Why might colour memory improve in breeding condition for females, but not for males (Figure 3.5)? One ultimate explanation could be that improved colour memory is related to mate choice. The plumage of male cowbirds that are nutritionally stressed has a lower brightness, hue, and saturation than the plumage of males that are fed *ad libitum* (McGraw et al. 2002). Females generally prefer to mate with males exhibiting the brightest ornaments (Olson and Owens 1998) and female colour memory during breeding may contribute to successful mate choice.

Another ultimate explanation may be that females use colour cues to find suitable host nests to parasitize. Although cowbirds are considered host generalists, they do show host selectivity (Lowther 1993, Woolfenden et al. 2003). In some populations, cowbirds parasitize preferred hosts first, but switch to less preferred hosts when others are unavailable (Woolfenden et al. 2003). Hosts themselves and host eggs vary in colour (Lowther 1993) and colour may influence nest selection.

A proximate explanation for better performance by females on the colour task in breeding condition could be that colour vision differs between the sexes and may vary with breeding condition. Female cowbirds have poorer chromatic visual resolution than males (Fernández-Juricic et al. 2013). Based on their time of capture, the birds examined by Fernández-Juricic et al. (2013) were probably in non-breeding condition. There may, thus, be differences between male and female cowbirds in colour vision and the nature of this difference may vary with breeding condition

3.4.3 Spatial versus colour memory

Both female and male cowbirds performed better on the spatial task than on the colour task (Figure 3.6). I showed that birds did not use a spatial strategy to solve the colour task early in the Progressive RI phase, several sessions before colour testing (Figure 3.7). This result suggests that the effect of order (spatial before colour) was minimalized or possibly eliminated with extensive practice on the colour task, although I cannot completely rule out potential order effects.

Better performance on the spatial task relative to the colour task may simply be the result of how spatial and colour memory was tested and not a general difference between spatial and colour memory in cowbirds. The colour task may have been more difficult because of the difference in perceptual space between the correct match and distractors, or the colour samples may have been less memorable. Alternatively, species that have strong demands for spatial memory may perform better on spatial than colour tasks. For example, food-storing black-capped chickadees (*Poecile atricapillus*) performed better on a spatial touchscreen task than on a colour touchscreen task whereas non-food-storing dark-eyed juncos (*Junco hyemalis*) performed equally well on both tasks (Brodbeck and Shettleworth 1995). Enhanced performance on the spatial task in chickadees was proposed to be related to the chickadees' reliance on memory for the location of stored food in the wild (Brodbeck and Shettleworth 1995). Other food-storing species are better than non-food-storers on spatial DMTS touchscreen tasks similar to mine (McGregor and Healy 1999). Free-living cowbirds, both males and females, may rely more heavily on spatial information than on colour information in behaviours resembling my DMTS task, such as foraging for seeds or invertebrates on the ground. It

is also possible that sex-specific selection acting on females for enhanced spatial memory has affected males. Many genes are obviously shared between the sexes and this genetic correlation can cause a trait favored in one sex to occur in both (Lande 1980, Wyman et al. 2013). A test of this hypothesis would be to compare performance of male and female icterids that are not brood parasites on the spatial and colour DMTS tasks and the orientation task used in (Chapter 2, Guigueno et al. 2014).

3.4.4 Conclusions

Sex differences in spatial memory abilities in brown-headed cowbirds depend on the task used to assess spatial ability. Females performed better than males in a related study in which birds moved through their testing environment to return to a remembered spatial location. However, male cowbirds performed better than females on a stationary spatial task in the current experiment. This task-dependent sex difference in spatial memory is the reverse of that observed in humans and could be due to trade-offs between different forms of spatial memory, differential selection, or functional incompatibility. Colour memory was found to improve in females from non-breeding condition to breeding condition and may play a role in mate choice or host nest selection. Finally, males and females alike were better on the spatial task than on the colour task, suggesting that memory for spatial information may be more accurate than memory for colour information in both sexes in some contexts in their natural environment.

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Chapter 4

4. Sex and seasonal changes in hippocampus volume and neurogenesis of brown-headed cowbirds and red-winged blackbirds⁴

4.1 Introduction

The brain can be specialized to maximize fitness by accommodating behavioural demands an organism routinely encounters in the wild (Sherry 2006, Smulders et al. 2010). This specialization has been demonstrated in the hippocampus, partly because the hippocampus is strongly associated with spatial memory and navigation, which are behaviours that can be easily measured. For instance, the hippocampus varies in size according to space use in a variety of organisms, and lesions of the hippocampus selectively disrupt spatial memory (Sherry and Vaccarino 1989, Hampton and Shettleworth 1996, Broadbent and Colombo 2000, Shiflett et al. 2003). In addition to hippocampus size, hippocampal neurogenesis may be involved in spatial memory,

⁴ This chapter has not been published. David Sherry and Scott MacDougall-Shackleton will be co-authors when this chapter is published as they were involved in the conceptual development, experimental design, and editing of the manuscript.

especially if an individual must encode new information about its surroundings (Barnea and Pravosudov 2011). An organism's environment is constantly changing and new neurons might help form new memories and replace old neurons that contain information from the past that may cause interference (Barnea and Pravosudov 2011). Thus, organisms that must rely more on spatial memory to reproduce and/or survive may have evolved a larger hippocampus with more neurogenesis.

The hippocampus of food-storing and migratory birds is frequently larger with greater hippocampal neurogenesis than non-food-storing and non-migratory birds. Birds belonging to families in which some species store food, such as Paridae and Sittidae, have a larger relative hippocampus than birds from non-food-storing families (Krebs et al. 1989, Sherry et al. 1989). Phylogenetic comparisons have shown that relative hippocampus size is positively related to food-storing behaviour (Garamszegi and Eens 2004, Lucas et al. 2004). Within a food-storing species, hippocampus size (Roth and Pravosudov 2009) and hippocampal neurogenesis (Chancellor et al. 2011) are greater in populations that depend more on stored food due to harsher environmental conditions (Roth and Pravosudov 2009). Migratory sub-species of white-crowned sparrows (*Zonotrichia leucophrys*) have a larger hippocampus (Pravosudov et al. 2006) and higher hippocampal neurogenesis (LaDage et al. 2011) than non-migratory sub-species. Hippocampal neurogenesis is also higher in a migratory Old World *Acrocephalus* warbler than a closely-related non-migratory species (Barkan et al. 2014). In sum, hippocampus size and neurogenesis are closely associated with patterns of space use across songbird species.

Although there are patterns of hippocampus size and neurogenesis relating to space use among species, seasonal changes in the hippocampus within species have yielded conflicting results. In chickadees, Smulders et al. (1995) reported a peak in hippocampus volume in October, which corresponds roughly to the first half of the food-storing season. In support, Barnea and Nottebohm (1994) reported that neurogenesis was also at its peak at that time. However, Hoshooley and Sherry (2004) used the exogenous cell birth marker bromodeoxyuridine (BrdU) and found no seasonal variation in the production of new neurons in the hippocampus. In addition, Hoshooley and Sherry (2004) found no seasonal variation in apoptosis of mature neurons. Thus, the results from Barnea and Nottebohm (1994) could only be explained by seasonal differences in the survival of new neurons. Likewise, subsequent studies were unable to replicate the results from Smulders et al. (1995). Two studies found no change in hippocampal volume throughout the year (Hoshooley and Sherry 2004, Hoshooley et al. 2007), while a third reported results opposite to those of Smulders et al. (1995) in which chickadees had a larger hippocampus from February to April than from October to November (Hoshooley and Sherry 2007). Sherry and MacDougall-Shackleton (2014) proposed that hippocampal volume and neurogenesis in chickadees, and thus discrepancies between studies, could be explained by the year-to-year food-storing experience of chickadees (food availability, flock size, temperatures, etc.). Unlike the hippocampus, there is a clear photoperiodically-induced pattern of neurogenesis in other brain regions, such as the song control nuclei (Tramontin and Brenowitz 2000). The song control nucleus HVC undergoes a peak in neurogenesis in the autumn when some species of songbirds modify their song for the upcoming breeding season (Tramontin and Brenowitz 1999, Nottebohm 2004). Clear

seasonal patterns of neurogenesis in the hippocampus do not seem to exist in the same manner as they do with HVC. However, seasonal changes in the volume and neurogenesis of the hippocampus have not been studied in birds other than food-storers.

In addition to the species and seasonal differences described above, sex differences in hippocampus size and neurogenesis have been reported in mammals. For instance, meadow voles (*Microtus pennsylvanicus*) are polygynous and males have home ranges that are 4-7 times larger than the home ranges of females, whereas there is no sex difference in space use in monogamous pine voles (*M. pinetorum*; Gaulin and FitzGerald 1986, 1989). Consistent with these sex and species differences in space use, the hippocampus is larger in male meadow voles than females, but no sex difference exists in pine voles (Jacobs et al. 1990). In Richardson's ground squirrels (*Urocitellus richardsonii*), which are also polygynous, males have a larger hippocampus with more neurogenesis than females (Burger et al. 2013, Burger et al. 2014). The hippocampus in mammals is evolutionarily homologous to that in birds (Colombo and Broadbent 2000, Jarvis et al. 2005). Thus, I would expect trends in birds to mirror those in mammals, and as 90% of birds are socially monogamous and provide bi-parental care (Mock and Fujioka 1990), I would expect few sex differences in hippocampus size or neurogenesis in bird species.

Although sex differences are not expected in most bird species, previous work on avian brood parasites has found sex differences in hippocampus size and spatial memory, in addition to seasonal differences in hippocampus size. In brown-headed cowbirds (*Molothrus ater*), only females repeatedly visit dozens of host nests per breeding season to assess the stage of completion of their clutches, to lay their own eggs, and to remove

host eggs (Norman and Robertson 1975, Rothstein et al. 1986, Gates and Evans 1998, White et al. 2009, Guigueno and Sealy 2011). Female cowbirds performed better than males on a spatial task requiring birds to find hidden food in a large room, with performance remaining stable across breeding conditions (Chapter 2, Guigueno et al. 2014). Female brown-headed cowbirds have also been found to have a larger hippocampus relative to the telencephalon than males (Sherry et al. 1993), however seasonal effects were not studied. A sex difference in hippocampus size was also observed in shiny cowbirds (*M. bonariensis*), a South American relative in which, like brown-headed cowbirds, only females search for nests (Reboreda et al. 1996). However, no sex differences were found in screaming cowbirds (*M. rufoaxillaris*), a brood parasite in which males assist in nest searching (Reboreda et al. 1996). In a seasonal comparison, (Clayton et al. 1997) both shiny and screaming cowbirds had a larger relative hippocampus size in the breeding season, but a sex difference only existed in shiny cowbirds. The sex difference in hippocampus size in favour of females found in shiny and brown-headed cowbirds is thus the reverse of that usually found in mammals. In sum, few studies have simultaneously examined sex and seasonal differences in the hippocampus size of brood parasites and none have incorporated hippocampal neurogenesis.

In the current study, I tested for potential sex differences and seasonal changes in hippocampal volume and neurogenesis in brown-headed cowbirds (hereafter “cowbirds”) and a close relative that is not a brood parasite, the red-winged blackbird (hereafter “blackbirds”, *Agelaius phoeniceus*). Like most other birds, blackbirds normally provide bi-parental care and females and males have similar home ranges (Yasukawa and Searcy

1995). I hypothesized that the hippocampus of cowbirds is specialized for their brood-parasitic mode of reproduction. Specifically, I predicted that cowbirds would have a larger hippocampus with more neurogenesis than blackbirds and that a sex difference would exist in cowbirds only, with female cowbirds having a larger hippocampus with more neurogenesis than male cowbirds. Due to conflicting results from studies on food-storers and other cowbird species, my seasonal predictions were uncertain. I predicted neurogenesis would be heightened in the autumn, like with song control nuclei. Because spatial performance of brown-headed cowbirds on a navigational task did not vary seasonally (Chapter 2, Guigueno et al. 2014), I predicted that relative hippocampus volume would remain stable between breeding conditions. However, in other organisms such as deer mice (*Peromyscus maniculatus*), a sex difference in spatial performance only existed in breeding condition when spatial memory load was at its highest (Galea et al. 1996) and South American cowbirds have a larger hippocampus in breeding condition (Clayton et al. 1997). Therefore, if the hippocampus volume was not seasonally stable as I predicted, I expected a female-biased sex difference to be greatest in breeding condition when female cowbirds search for host nests in the wild.

4.2 Methods

4.2.1 Subjects

Female and male cowbirds and blackbirds were collected in breeding and post-breeding conditions (Table 4.1). I collected birds in breeding condition between mid-

Table 4.1. Number of brains collected from each experimental group. Brains were collected the day after the birds were captured from the field.

Breeding condition	Brown-headed cowbird		Red-winged blackbird	
	Female	Male	Female	Male
Breeding	15	16	8	16
Post-breeding	8	8	8	8

March and mid-May 2013 and birds in post-breeding condition between mid-September and mid-November 2013. All birds were captured using ground traps and mist nets at various sites near Port Rowan, Ontario, Canada. Mean body weights \pm SE were as follows: 39.62 g \pm 0.63 (female cowbirds; $n = 22$), 50.33 g \pm 0.97 (male cowbirds; $n = 23$), 42.49 g \pm 0.66 (female blackbirds; $n = 16$), and 65.29 g \pm 0.98 (male blackbirds; $n = 23$). After capture, birds were transported to the Advanced Facility for Avian Research at the University of Western Ontario where they were housed overnight in individual cages with food and water.

4.2.2 Blood sampling

I collected blood samples in the field to confirm breeding condition. I punctured the brachial vein of each bird with a 26-gauge needle. I collected all the samples within 30 min, except for 14 out of 88 samples that were taken 30-92 minutes after capture. There was no statistically significant correlation between androgen concentration and time, therefore I kept these data points in the analyses. I collected approximately 400 μ L of blood into heparinized capillary tubes and centrifuged the blood for 10 min at 13,000 \times g. Finally, I extracted the plasma from the tubes with a Hamilton syringe and froze the plasma at -30 $^{\circ}$ C until the hormone assay.

4.2.3 Androgen assay

Testosterone increases in the breeding season for both female and male brown-headed cowbirds (Chapters 2 and 3; Dufty and Wingfield 1986 a,b). I assayed plasma androgen concentration using a testosterone enzyme immunoassay previously validated for a variety of bird species (EIA; Cat. #1-2402, Salimetrics; [Washburn et al. 2007]). I

previously validated the assay in cowbirds (see Chapters 2 and 3). To validate the assay for blackbirds, I used the same protocol as in Chapters 2 and 3 and Newman et al. (2008) and I assayed a serial dilution of blackbird plasma and compared measured levels of testosterone in the dilutions to the standard curve using an ANCOVA. A non-significant interaction term ($F_{1,10} = 0.01$, $p = 0.94$) indicated that the slopes were similar and that the assay was suitable for blackbirds. Intra-assay variation was 8.85%. Inter-plate variation, based on a pooled red-winged blackbird plasma sample and low and high controls was 3.79%. The sensitivity of the assay was 5 pg/mL (two standard deviations from the average value of zero on the standard curves). Samples below this level were assigned a value of 2.5 pg/mL for the analyses.

4.2.4 Brain collection

The day after capture, I deeply anesthetized the birds using isoflurane. I then transcardially perfused the birds with heparinized saline, followed by 4% paraformaldehyde. The brains were removed from the skull and then placed in 4% paraformaldehyde for 24 h, followed by 30% sucrose in phosphate-buffered saline (PBS) for 48-72 h (until the brains sunk to the bottom of the vial). Finally, I froze the brains on crushed dry ice and stored them in aluminum foil at -80 °C until the start of immunohistochemistry.

4.2.5 Immunohistochemistry

I sectioned the brains into 40 μ m coronal sections using a cryostat. Two alternating sets of brain sections, each set ten sections apart, were collected for NeuN and doublecortin (DCX) immunoreactivity. NeuN is a protein expressed in most mature

neurons (Mullen et al. 1992) and was used to calculate the volume of the hippocampus. To measure hippocampal neurogenesis, I examined DCX immunoreactivity. DCX is a microtubule-associated endogenous protein only expressed in migrating and differentiating immature neurons (Francis et al. 1999, Gleeson et al. 1999) and is a reliable marker of neurogenesis (Balthazart and Ball 2014a,b). Each run consisted of a random sample of brains from different groups (Table 4.1).

I used the following steps for NeuN immunohistochemistry. First, I washed free-floating sections twice in 0.1M PBS then incubated the sections in 0.5% hydrogen peroxide for 30 min. Next, I washed sections three times in PBS then incubated the sections in 10% normal goat serum (Vector, Burlingame, CA, USA) that was diluted in 0.3% Triton in PBS (PBST) overnight. I replaced the diluted goat serum with primary monoclonal antibody (made in mouse, MB377, Millipore, Billerica, MA, USA) diluted 1:2000 in 0.3% PBST and incubated overnight. Next, I washed the sections three times in 0.1% PBST and incubated the sections for 1 h in biotinylated secondary antibody (goat anti-mouse IgG, Vector) diluted 1:250 in 0.3% PBST. Then, I washed the sections three times in 0.1% PBST and incubated the sections for 1 h in avidin-biotin horseradish-peroxidase complex (VectaStain Elite ABC Kit) diluted 1:200. I washed the sections three times in 0.1% PBST and visualized the sections by exposing them to diaminobenzidine solution (Sigma Fast-DAB), followed by four washes in PBS. I mounted the sections onto gelatin-coated slides, dehydrated them gradually with increasing ethanol concentrations, cleared them in solvent (Harleco Neo-Clear, EMD Chemicals, Billerica, MA, USA), and cover slipped the slides using Permount (Fisher Scientific, Pittsburgh, PA, USA).

The DCX immunohistochemistry protocol was similar to the NeuN protocol, except for the following differences. First, the sections were incubated in 0.5% hydrogen peroxide for 15 min instead of 30 min. Second, the sections were incubated in 10% normal horse serum (Vector, Burlingame, CA, USA) instead of goat serum and were incubated for 1 h instead of overnight. Finally, the primary antibody that I used was made in goat against DCX (polyclonal, Santa Cruz Biotechnology, Santa Cruz, CA, USA) diluted 1:250 and the secondary was biotinylated horse anti-goat IgG (Vector) diluted 1:400.

4.2.6 Microscopy

I used the NeuN-labelled sections to measure the volume of the hippocampus (Figure 4.1). I identified the boundary of the hippocampus by the presence of different cell densities and cell sizes relative to the hyperpallium apicale (HA), a region adjacent and lateral to the hippocampus. I captured images of the hippocampus with a Spot Idea 5-megapixel digital camera (Diagnostics Instruments) mounted on a Zeiss Axiophot microscope using a 1.25X objective lens. Only bird ID was assigned to each photo, therefore the images were analyzed without reference to sex, species, or season. The perimeter of the hippocampus was traced in ImageJ software (NIH). I summed the frusta volumes (truncated cone) between sections (400 μm) to estimate the total volume of hippocampus in both hemispheres. I captured images of the telencephalon with a high-resolution (2400 dpi) flatbed scanner with a transparency adapter and I traced the perimeter of every tenth telencephalon section with ImageJ. I used the frustum formula

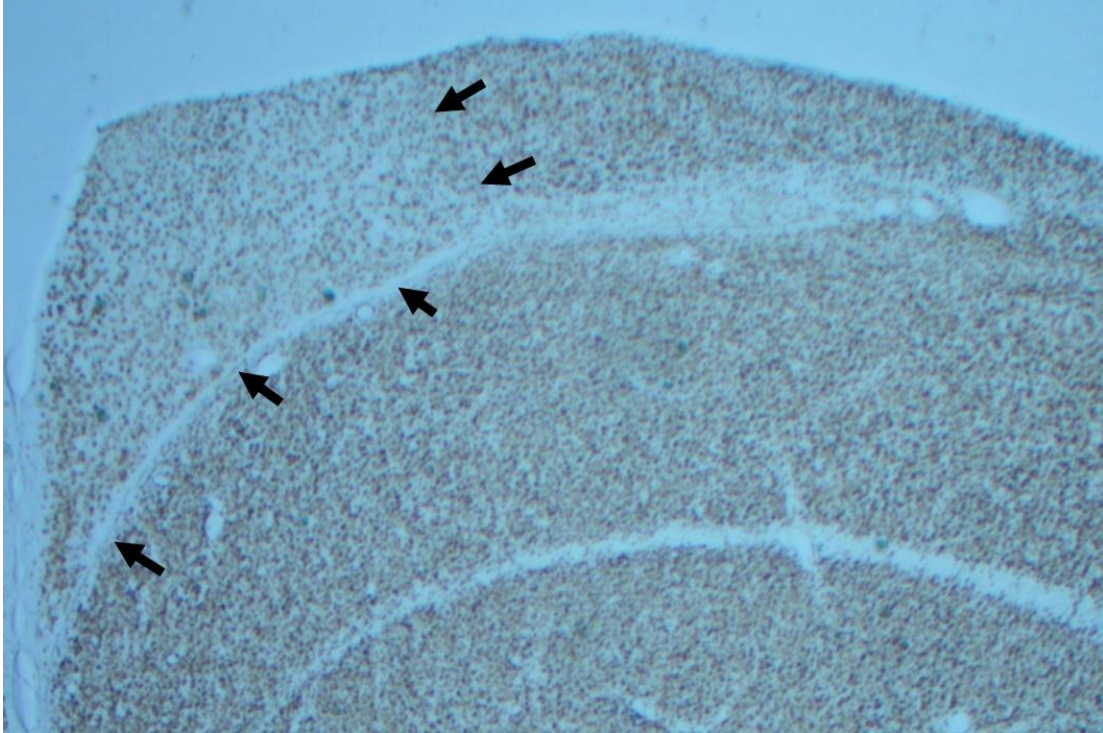


Figure 4.1. NeuN staining used for volume analyses, with the hippocampus boundary indicated by arrows.

and summed the frusta volumes between each tissue section (400 μm) to estimate total telencephalon volume in both hemispheres. The hippocampus and telencephalon volumes used in the analyses for each bird were the average between hemispheres. I adjusted the sampling interval and used the next nearest section if a section was damaged or lost. Sample sizes for volume analyses are found in Table 4.1. I used DCX-labelled sections to quantify immunoreactivity, which was interpreted as neurogenesis (Figure 4.2). I captured images of DCX immunoreactive (DCX+) round and fusiform cells and projections with a Leica DFC 420C camera mounted to a Leica DM5500B microscope (Figure 4.2). I chose three sections (rostral, medial, and caudal) from the hemisphere that was most intact and best stained. The rostral section was located 800-1200 μm after the rostral edge of the hippocampus (moving rostro-caudally through the brain) and the caudal section was located 1200-2000 μm before the caudal end of the hippocampus. The beginning and end of the hippocampus were identified by searching for a lack of a boundary between hippocampus and HA as described above. The medial section was chosen to be in between and approximately equidistant to the rostral and caudal sections, near the coronal plane of the anterior commissure. For each section, I chose the following sampling fields: 1) three fields inside the hippocampus; one in the ventral position, the second in the medial position, dorsal to the ventral field of view (as in Wada et al. 2014), and the third lateral to the medial field of view (medial section only), 2) two fields outside the hippocampus to serve as covariates for the interior hippocampus analyses; one in the HA and one in the telencephalon, lateral to the interior hippocampus ventral field of view and 3) three sampling fields in the subventricular zone (SVZ) in the ventral, medial, and lateral (medial section only) positions, which included half of the field of

Figure 4.2 . (A) Doublecortin staining, used to quantify neurogenesis, with the fields of view used in the analyses. Images were captured inside the hippocampus, with the hyperpallium apicale (HA) and the telencephalon (Tel) as covariates, and in the subventricular zone (SVZ), with the half of each field of view (telencephalon) acting as the covariate. V-Ventral, M-Medial, L-Lateral. (B) An example of a field of view in the SVZ, with the hippocampus in the upper half (dependent variable) and the telencephalon (covariate) in the lower half of the image. (C) Example of thresholding to measure the % doublecortin immunoreactive cover within a field of view. (D) Examples of round (top arrow) and fusiform (bottom arrow) cells.

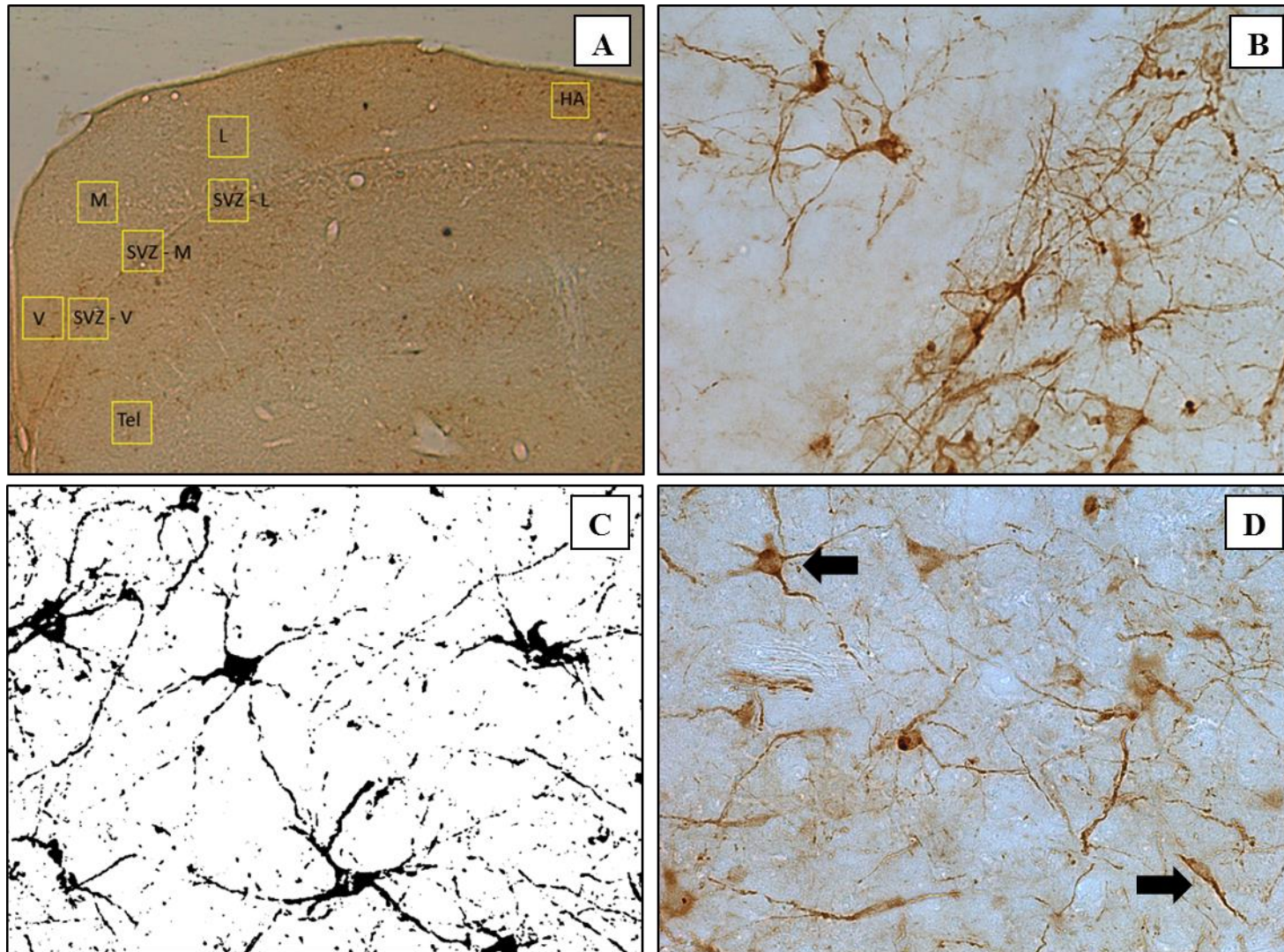


Figure 4.2

For each field of view from the DCX images, I captured z-stack images in 0.63 μm steps through the focal planes with a 40X objective lens. These images were then compiled using the montage mode in the Leica Application Suite software, which produced an image that displayed all DCX+ cells and projections in focus. I used the threshold feature in ImageJ to calculate the percent coverage by DCX+ cells and projections. Both fusiform and round cells were present, which are normally interpreted as migrating and recently differentiated neurons, respectively (Balthazart and Ball 2014a,b). Thus, I counted and analyzed these cell types separately. I was not able to quantify DCX+ in some birds due to poor staining, therefore sample sizes differ from Table 4.1 in the two following groups: breeding blackbird males ($n = 14$) and post-breeding cowbird males ($n = 6$).

4.2.7 Data analysis

All statistical analyses were performed in SAS (version 9.3, SAS Institute Inc., Cary, NC, USA). I used different analyses, depending on the data collected.

For the androgen analysis, I used a general linear model (PROC GLM) with species, sex, and breeding condition, and all interactions as explanatory variables, with androgen concentration as the dependent variable. I log-transformed the androgen concentrations to produce normally-distributed residuals.

For the volume analyses, I used a general linear model (PROC GLM) with species, sex, breeding condition, and all interactions as explanatory variables, telencephalon volume (minus the hippocampus) as a covariate, and hippocampus volume as the dependent variable. I also tested for volume changes in the telencephalon. I used a

general linear model (PROC GLM) with species, sex, breeding condition, and all interactions as explanatory variables. I log-transformed the volume data to produce normally-distributed residuals if they were not already normally distributed.

For the DCX analyses, the fields of view inside the hippocampus (ventral, medial, and lateral) were assigned the HA and telencephalon fields of view as covariates and were analyzed separately from the fields of view in the SVZ. The covariate (HA or telencephalon control) that produced the lowest AIC value was included in the final model. The telencephalon half of the SVZ field of view was the covariate for the SVZ analyses. Subject was set as a random factor and the explanatory variables were species, sex, breeding condition, area sampled and all interactions. To analyze % DCX+ cover, I used a linear mixed model (PROC MIXED) because I took multiple measurements from each subject (see details above). I log arcsine square root transformed proportions from the %DCX+ cover data. For the round cell analyses, I used generalized linear mixed models (PROC GLIMMIX) and specified a Poisson distribution as I had count data, which most frequently fits a Poisson distribution. For fusiform cell analyses, the model did not converge because some brain areas regions had too many zero data points (leading to a standard deviation and mean of zero for certain regions for many sexes and species). I therefore merged data from all areas and ran a general linear model with a Poisson link function (PROC GENMOD).

Although data were transformed for analyses, data were not transformed in the figures displaying the raw means \pm SE to ease visualization. However, figures displaying significant interactions for the DCX analyses show least squares means \pm SE of the

transformed data. Significant interactions were further analyzed using Fisher's LSD post-hoc tests. Results were considered significant if $p \leq 0.05$.

4.3 Results

4.3.1 Androgens

There was a significant main effect of breeding condition, with higher androgen levels for birds in breeding than post-breeding condition, and a significant main effect of sex, with males showing higher levels than females (Table 4.2, Figure 4.3). Thus, cowbirds were in the correct breeding condition. Species and all other interactions were non-significant (Table 4.2).

4.3.2 Volume

For relative hippocampus size, there was a significant main effect of species, with cowbirds having a larger hippocampus than blackbirds and a significant main effect of sex, with females having a larger hippocampus, relative to the telencephalon size, than males (Table 4.3, Figures 4.4A, 4.5). Breeding condition and all interactions were non-significant (Table 4.3). Once breeding and post-breeding data were merged, female cowbirds had the largest hippocampus of all four groups (female cowbirds, male cowbirds, female blackbirds, and male blackbirds), with female cowbirds having a larger hippocampus than male cowbirds (one-tailed post-hoc test: $t_{82} = 1.64$, $p = 0.05$), but female blackbirds also having a larger hippocampus than male blackbirds ($t_{82} = 2.07$, $p = 0.02$; Figures 4.4, 4.5).

Table 4.2. Summary of statistical effects of species, sex, breeding condition (BC) and their interactions on the concentration of circulating androgens. Results are from a general linear model. Significant effects are in bold.

Factors	<i>F</i>	d.f.	<i>p</i>-value
Androgens			
Species	3.10	1,79	0.08
Sex	3.85	1,79	0.05
BC	24.55	1,79	<0.0001
Species x Sex	0.32	1,79	0.57
Species x BC	0.29	1,79	0.59
Sex x BC	3.04	1,79	0.08
Species x Sex x BC	0.19	1,79	0.67

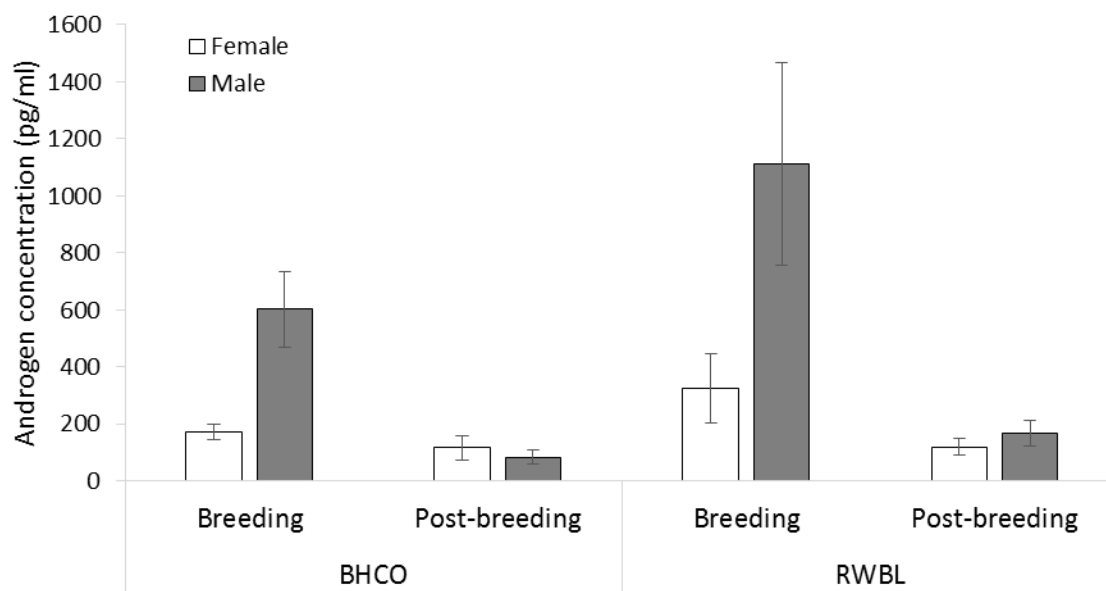
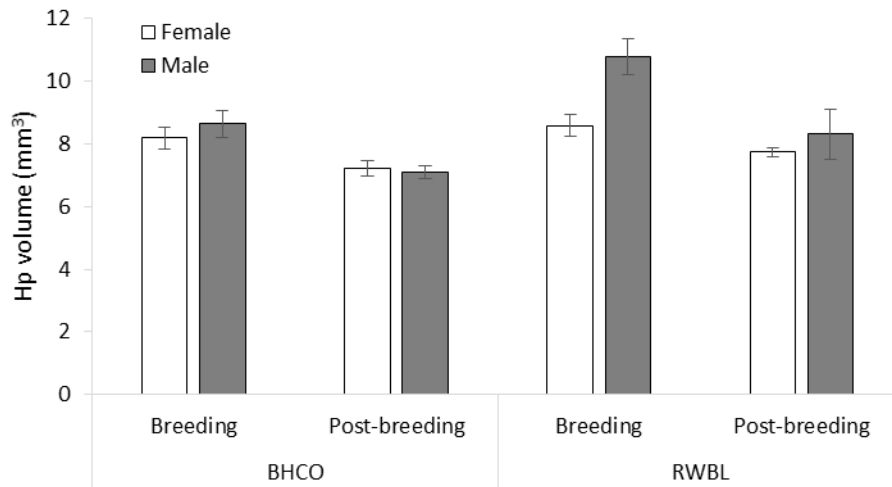


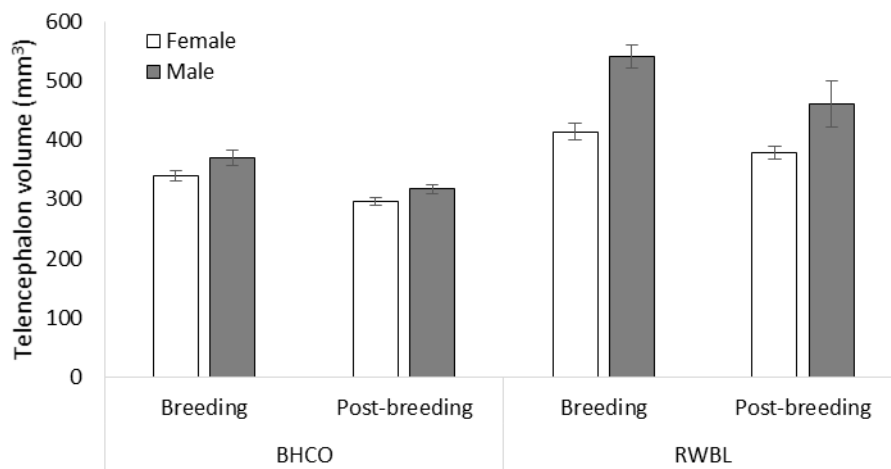
Figure 4.3. Mean \pm SE androgen concentrations in female and male brown-headed cowbirds (BHCO) and red-winged blackbirds (RWBL) in breeding and post-breeding conditions.

Table 4.3. Summary of statistical effects of species, sex, breeding condition (BC) and their interactions on the volume of the hippocampus and the telencephalon. Results are from a general linear model. Significant effects are in bold.

Factors	<i>F</i>	d.f.	<i>p</i>-value
Hippocampus volume			
Species	19.10	1,78	<0.0001
Sex	6.17	1,78	0.01
BC	0.79	1,78	0.38
Species x Sex	0.32	1,78	0.57
Species x BC	0.68	1,78	0.41
Sex x BC	1.35	1,78	0.25
Species x Sex x BC	0.09	1,78	0.76
Telencephalon (covariate)	91.53	1,78	<0.0001
Telencephalon volume			
Species	96.82	1,79	<0.0001
Sex	24.40	1,79	<0.0001
BC	21.82	1,79	<0.0001
Species x Sex	5.53	1,79	0.02
Species x BC	0.01	1,79	0.90
Sex x BC	0.89	1,79	0.35
Species x Sex x BC	0.45	1,79	0.50



A



B

Figure 4.4. Mean \pm SE hippocampus (Hp) and telencephalon volumes of brown-headed cowbirds (BHCO) and red-winged blackbirds (RWBL) and breeding and post-breeding conditions.

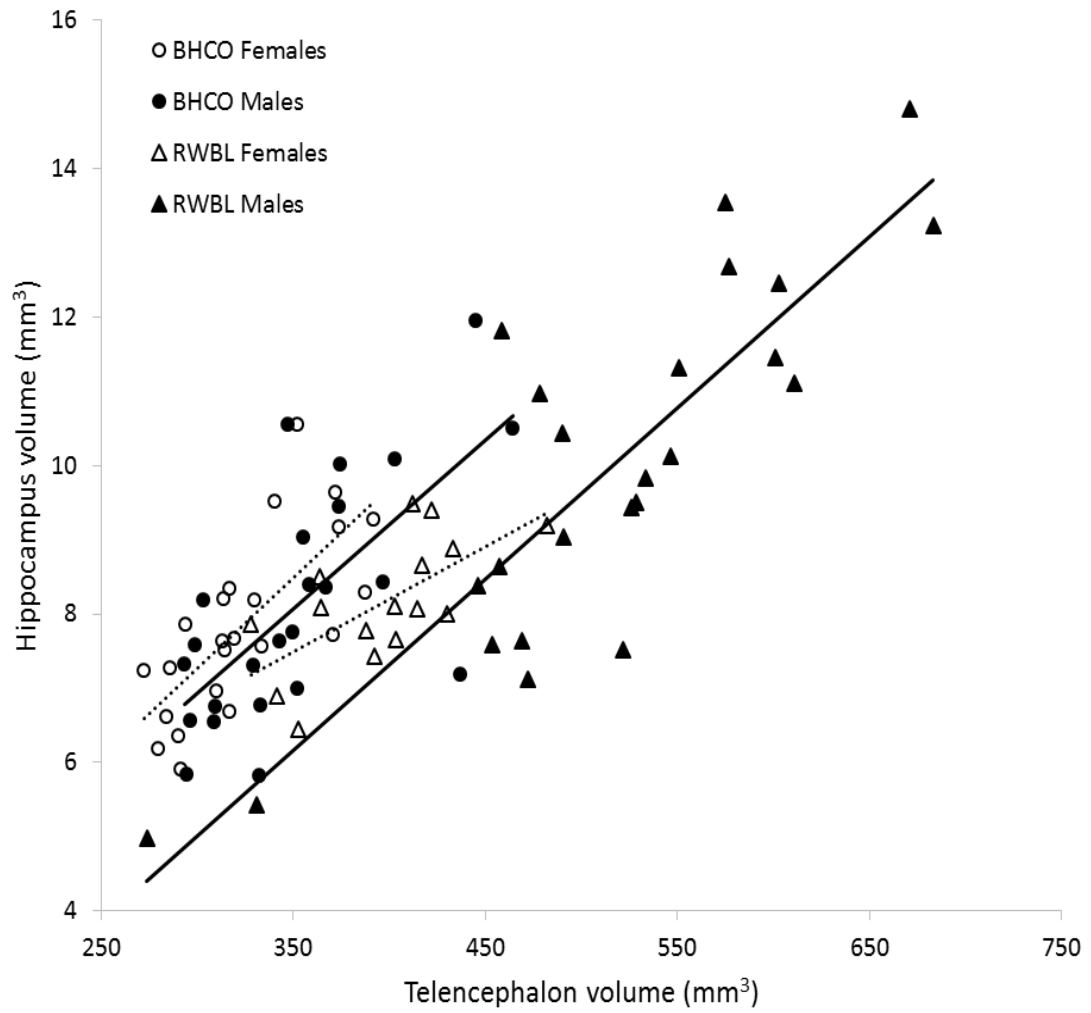


Figure 4.5. Hippocampus volume relative to the telencephalon volume in female and male brown-headed cowbirds (BICO) and red-winged blackbirds (RWBL). The trendlines fits the data as follows: top dotted line - female BICO, lower dotted line - female RWBL, top solid line - male BICO, and bottom solid trendline - male RWBL.

For the telencephalon, there were significant main effects of 1) breeding condition, with birds having a smaller telencephalon in post-breeding condition, 2) sex, with males having a larger telencephalon than females, and 3) species, with blackbirds having a larger telencephalon than cowbirds (Table 4.3, Figure 4.3B). However, a significant sex by species interaction indicated that the effects of sex and species above were mainly driven by the blackbirds, with male blackbirds having a larger telencephalon than female blackbirds ($t_{79} = 4.98$, $p < 0.0001$), but male cowbirds only had a nearly significantly larger telencephalon than female cowbirds ($t_{79} = 5.70$, $p = 0.06$).

4.3.3 Doublecortin

Data from neurogenesis analyses (Tables 4.4-4.8) are presented in detailed format by area (Figures 4.6-4.11) and by significant interactions between sex, species, and breeding condition (Figures 4.12-4.13).

Percent DCX+ cover

Inside the hippocampus, there was a significant effect of area, with the ventral fields of view in the medial and caudal sections having the highest immunoreactivity, relative to outside the hippocampus (Table 4.4, Figure 4.6). There was a significant species by breeding condition interaction, with immunoreactivity being greater in post-breeding condition in cowbirds ($t_{75} = 1.97$, $p = 0.05$), but greater in breeding condition in blackbirds ($t_{75} = 2.00$, $p = 0.05$) (Table 4.4, Figures 4.6, 4.12). There was also a significant species by area interaction, with cowbirds having higher immunoreactivity than blackbirds in the ventral field of view in the medial section ($t_{449} = 3.33$, $p = 0.0009$) (Table 4.4, Figure 4.6). Finally, there was a significant sex by breeding condition by area

Table 4.4 Summary of statistical effects of species, sex, breeding condition (BC), area and their interactions on the percent doublecortin (DCX) coverage inside the hippocampus (Hp). Results are from a linear mixed model. Significant effects are in bold.

Factors	<i>F</i>	d.f.	<i>p</i>-value
%DCX+ cover inside Hp			
Species	2.83	1,75	0.10
Sex	1.95	1,75	0.17
BC	0.01	1,75	0.94
Area	83.86	6,449	<0.0001
Species x Sex	1.09	1,75	0.30
Species x BC	8.00	1,75	0.006
Species x Area	2.78	6,449	0.01
Sex x BC	0.75	1,75	0.39
Sex x Area	1.03	6,449	0.41
BC x Area	0.71	6,449	0.64
Species x Sex x BC	1.38	1,75	0.24
Species x Sex x Area	0.80	6,449	0.57
Sex x BC x Area	2.25	6,449	0.04
Species x BC x Area	1.53	6,449	0.17
Species x BC x Area x Sex	1.48	12,449	0.18
ControlTel (covariate)	37.54	1,449	<0.0001

Table 4.5 Summary of statistical effects of species, sex, breeding condition (BC), area and their interactions on the % doublecortin immunoreactive (DCX+) cover in fields of view in the subventricular zone (SVZ). Results are from a linear mixed model.

Significant effects are in bold.

Factors	<i>F</i>	d.f.	<i>p</i>-value
%DCX+ cover in SVZ			
Species	16.07	1,75	0.0001
Sex	0.88	1,75	0.35
BC	0.30	1,75	0.59
Area	21.00	6,449	<0.0001
Species x Sex	4.35	1,75	0.04
Species x BC	10.96	1,75	0.001
Species x Area	2.17	6,449	0.05
Sex x BC	0.08	1,75	0.77
Sex x Area	1.21	6,449	0.30
BC x Area	0.75	6,449	0.61
Species x Sex x BC	0.68	1,75	0.41
Species x Sex x Area	0.88	6,449	0.51
Sex x BC x Area	1.90	6,449	0.08
Species x BC x Area	1.51	6,449	0.17
Species x BC x Area x Sex	1.25	12,449	0.28
Telencephalon in SVZ(covariate)	34.35	1,449	<0.0001

Table 4.6 Summary of statistical effects of species, sex, breeding condition (BC), area and their interactions on the number of round cells per field of view in the hippocampus (Hp). Results are from a generalized linear mixed model with a Poisson distribution. Significant effects are in bold.

Factors	<i>F</i>	d.f.	<i>p</i>-value
# round cells in Hp			
Species	0.64	1,75	0.43
Sex	1.04	1,75	0.31
BC	3.09	1,75	0.08
Area	21.17	6,449	<0.0001
Species x Sex	1.01	1,75	0.32
Species x BC	0.01	1,75	0.94
Species x Area	1.55	6,449	0.16
Sex x BC	0.08	1,75	0.78
Sex x Area	1.79	6,449	0.10
BC x Area	1.76	6,449	0.11
Species x Sex x BC	0.59	1,75	0.44
Species x Sex x Area	1.74	6,449	0.11
Sex x BC x Area	1.77	6,449	0.10
Species x BC x Area	1.02	6,449	0.41
Species x BC x Area x Sex	1.47	12,449	0.19
ControlHA (covariate)	19.71	1,449	<0.0001

Table 4.7 Summary of statistical effects of species, sex, breeding condition (BC), area and their interactions on the number of round cells per field of view in the subventricular zone (SVZ). Results are from a generalized linear mixed model with a Poisson distribution. Significant effects are in bold.

Factors	<i>F</i>	d.f.	<i>p</i>-value
# round cells in SVZ			
Species	0.1	1,75	0.75
Sex	0.71	1,75	0.40
BC	0.62	1,75	0.43
Area	5.53	6,449	<0.0001
Species x Sex	1.94	1,75	0.17
Species x BC	2.74	1,75	0.10
Species x Area	2.2	6,449	0.04
Sex x BC	0	1,75	0.97
Sex x Area	0.36	6,449	0.90
BC x Area	1.93	6,449	0.07
Species x Sex x BC	0.82	1,75	0.37
Species x Sex x Area	0.68	6,449	0.66
Sex x BC x Area	0.67	6,449	0.67
Species x BC x Area	1.03	6,449	0.40
Species x BC x Area x Sex	0.41	12,449	0.87
Telencephalon in SVZ(covariate)	8.28	1,449	0.004

Table 4.8 Summary of statistical effects of species, sex, breeding condition (BC), area and their interactions on the number of fusiform cells per field of view in the hippocampus (Hp). Results are from a Poisson regression analysis. Significant effects are in bold.

Factors	χ	d.f.	<i>p</i>-value
# fusiform cells in Hp			
Species	6.54	1	0.01
Sex	5.27	1	0.02
BC	2.06	1	0.15
Species x Sex	5.23	1	0.02
Species x BC	0.12	1	0.73
Sex x BC	1.05	1	0.31
Species*Sex*BC	1.56	1	0.21
ControlTel (covariate)	13.55	1	0.0002

Table 4.9 Summary of statistical effects of species, sex, breeding condition (BC), area and their interactions on the number of fusiform cells per field of view in the subventricular zone (SVZ). Results are from a Poisson regression analysis. Significant effects are in bold.

Factors	χ	d.f.	<i>p</i>-value
# fusiform cells in SVZ			
Species	4.40	1	0.04
Sex	1.50	1	0.22
BC	4.61	1	0.03
Species x Sex	3.85	1	0.05
Species x BC	0.53	1	0.47
Sex x BC	3.54	1	0.06
Species*Sex*BC	2.19	1	0.14
Telencephalon in SVZ(covariate)	34.24	1	<0.0001

Figure 4.6. Mean % doublecortin immunoreactive (DCX+) cover \pm SE in fields of view inside the hippocampus (Hp) with covariates in the telencephalon (ControlTel) and the hyperpallium apicale (ControlHA) in the rostral, medial, and caudal regions of the brains of female and male brown-headed cowbirds and red-winged blackbirds in breeding and post-breeding conditions.

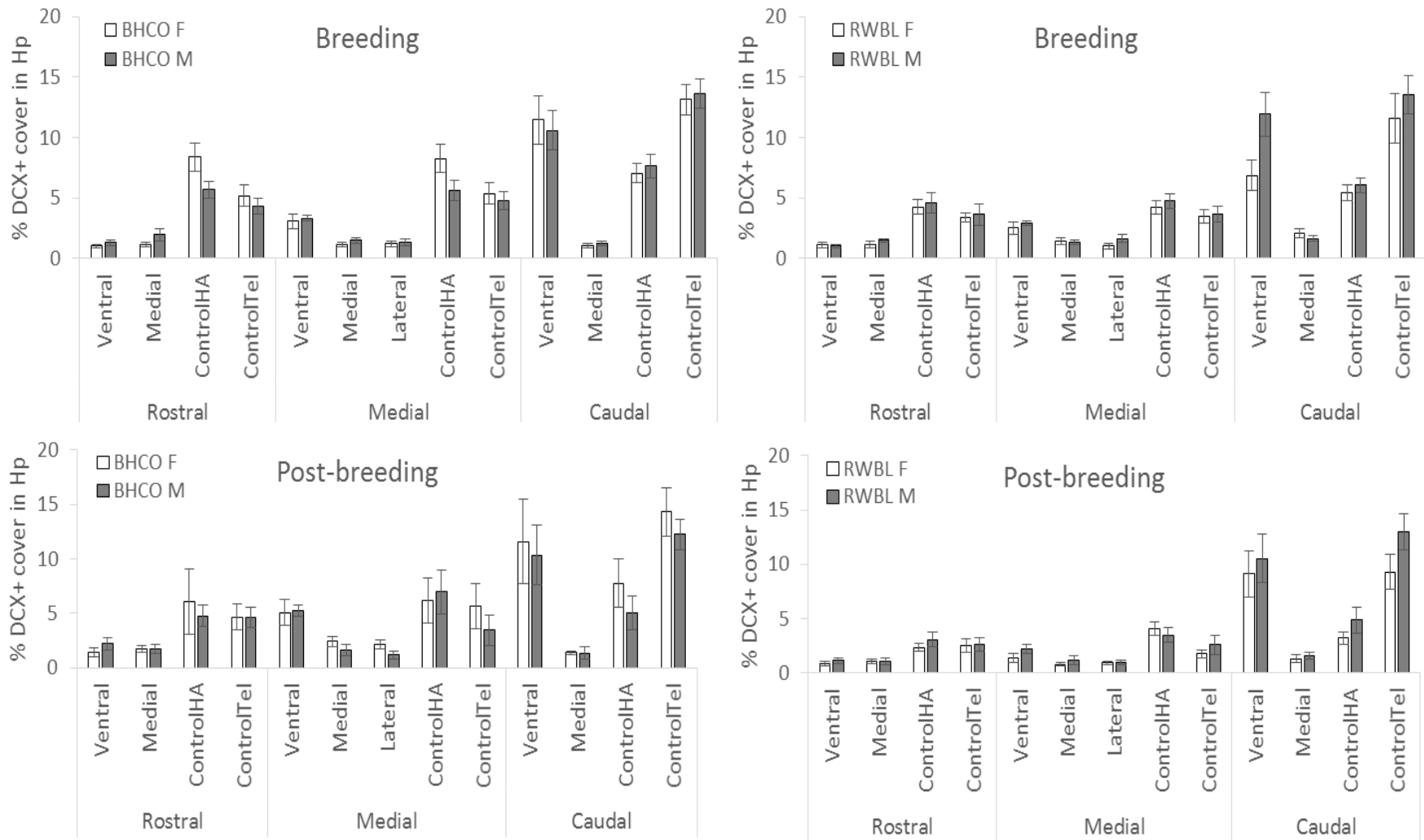


Figure 4.6

Figure 4.7. Mean % doublecortin immunoreactive (DCX+) cover \pm SE in fields of view in the subventricular zone (SVZ), with the hippocampus (Hp) edge acting as the dependent variable and the telencephalon (Tel) edge acting as the covariate in the rostral, medial, and caudal regions of the brains of female and male brown-headed cowbirds and red-winged blackbirds in breeding and post-breeding conditions.

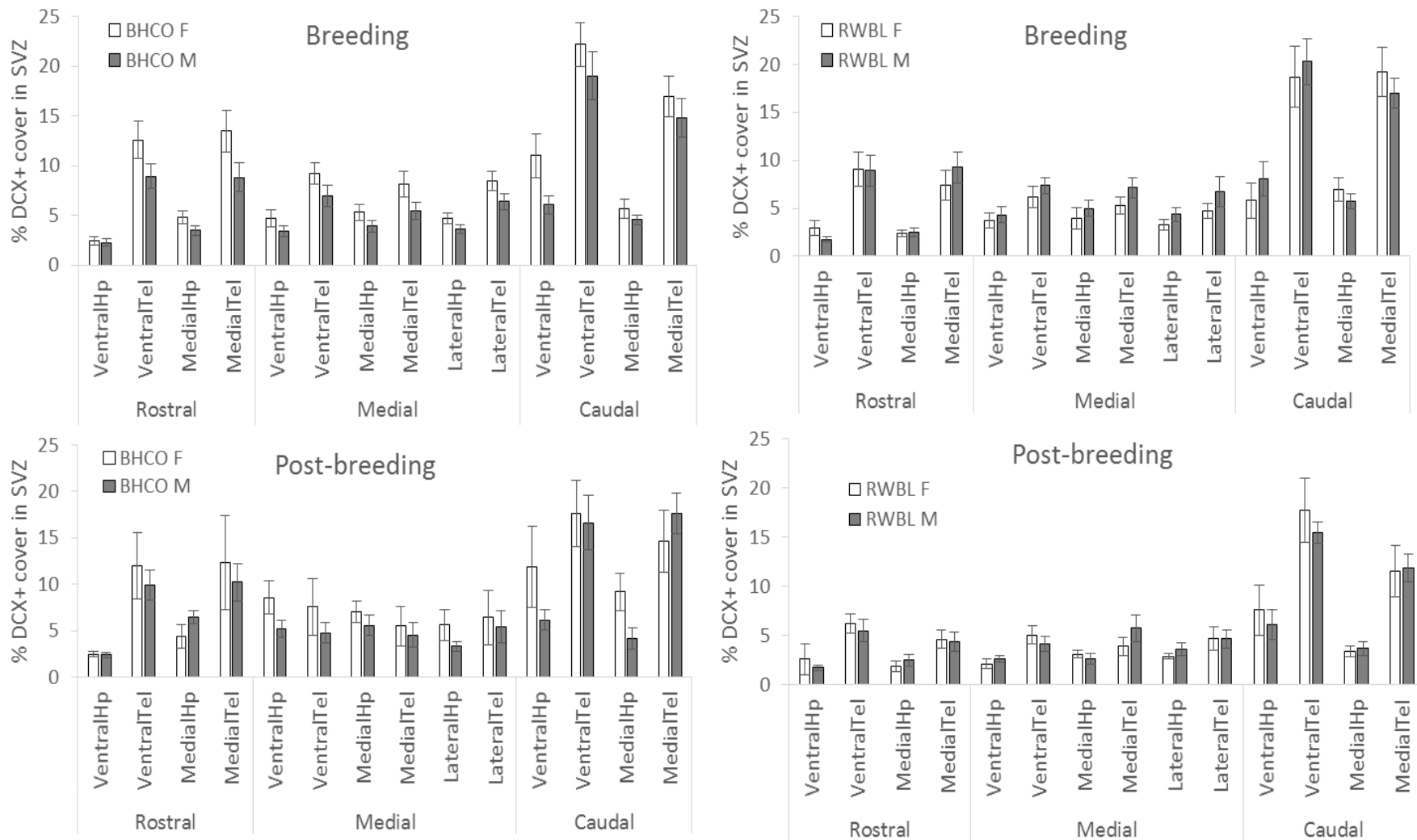


Figure 4.7

Figure 4.8. Mean number of round cells \pm SE per field of view inside the hippocampus (Hp) with covariates in the telencephalon (ControlTel) and the hyperpallium apicale (ControlHA) in the rostral, medial, and caudal regions of the brains of female and male brown-headed cowbirds and red-winged blackbirds in breeding and post-breeding conditions.

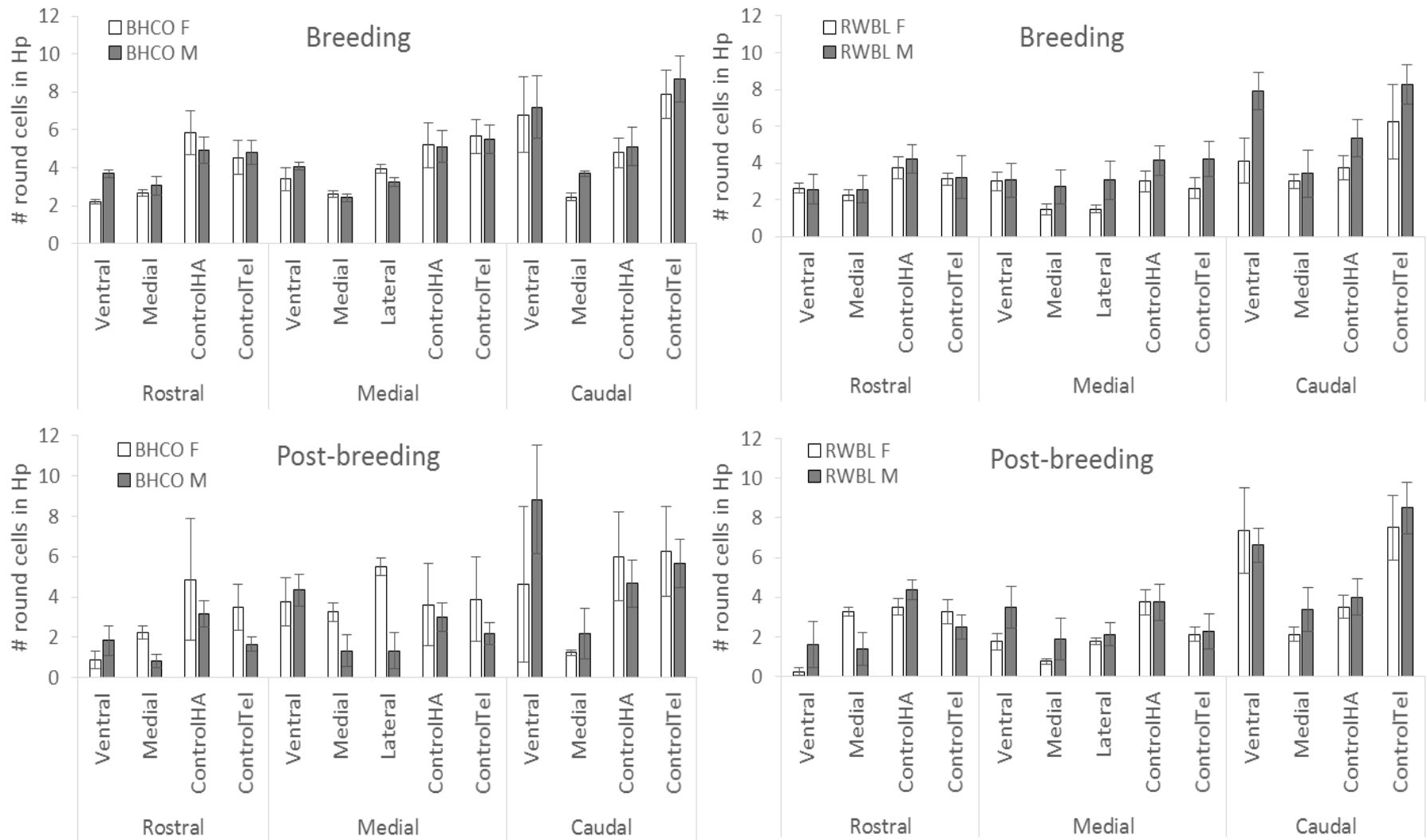


Figure 4.8

Figure 4.9. Mean number of round cells \pm SE per field of view in the subventricular zone (SVZ), with the hippocampus (Hp) edge acting as the dependent variable and the telencephalon (Tel) edge acting as the covariate in the rostral, medial, and caudal regions of the brains of female and male brown-headed cowbirds and red-winged blackbirds in breeding and post-breeding conditions.

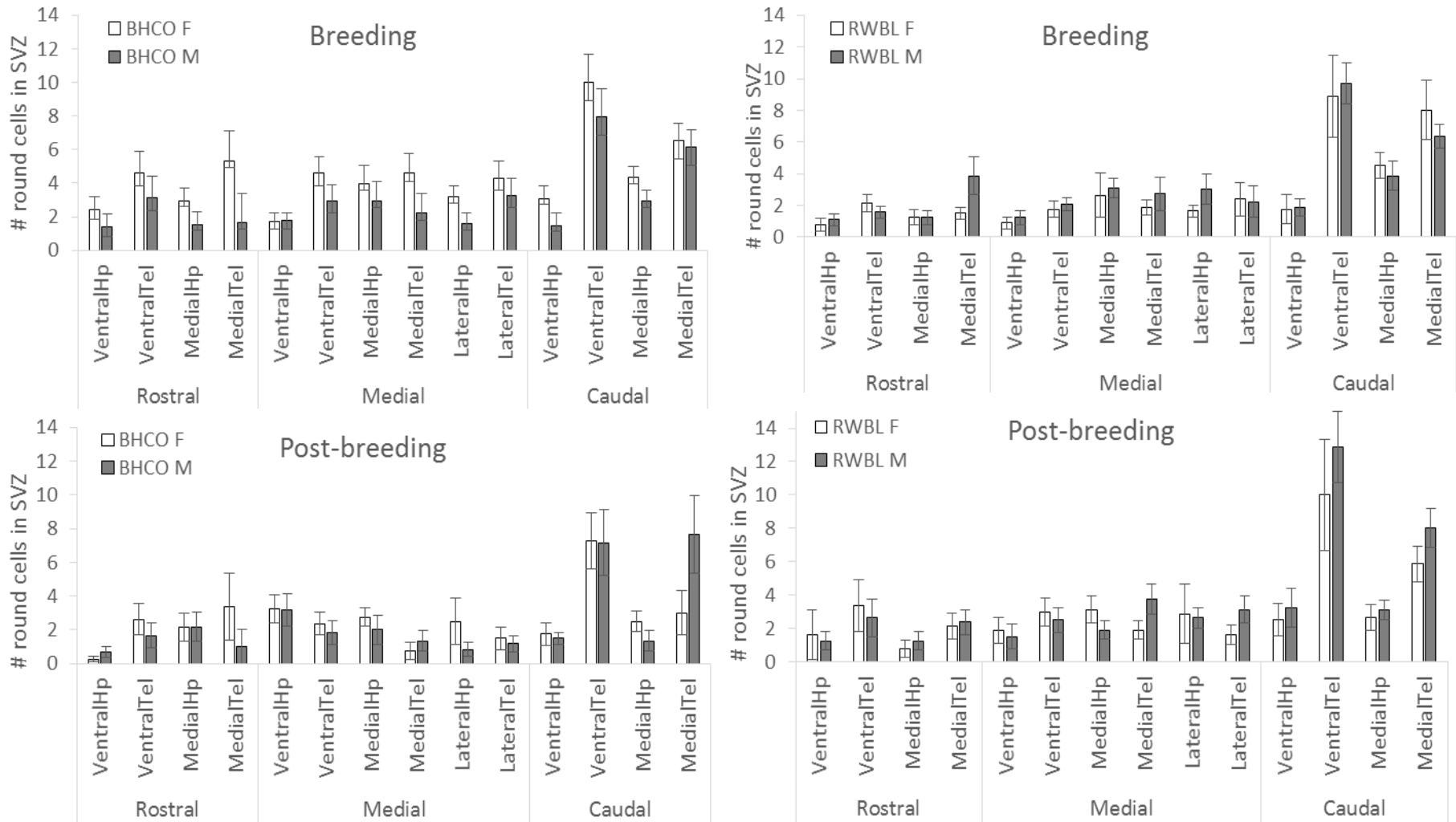


Figure 4.9

Figure 4.10. Mean number of fusiform cells \pm SE per field of view inside the hippocampus (Hp) with covariates in the telencephalon (ControlTel) and the hyperpallium apicale (ControlHA) in the rostral, medial, and caudal regions of the brains of female and male brown-headed cowbirds and red-winged blackbirds in breeding and post-breeding conditions.

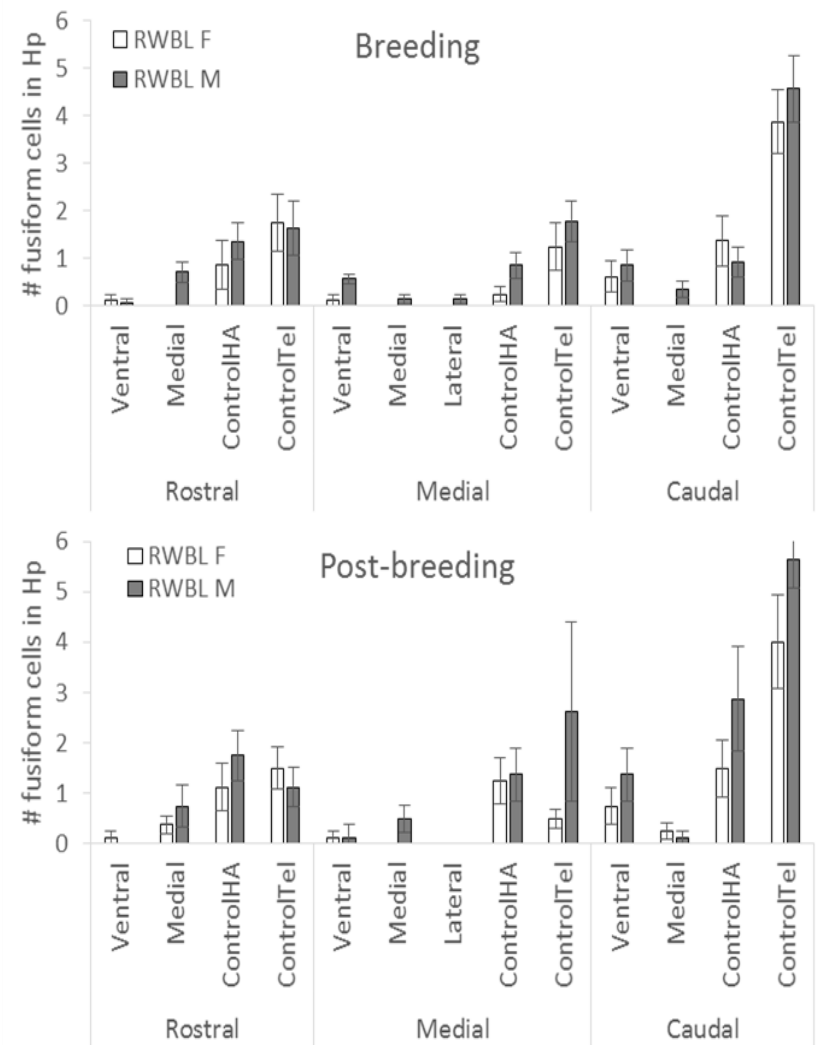
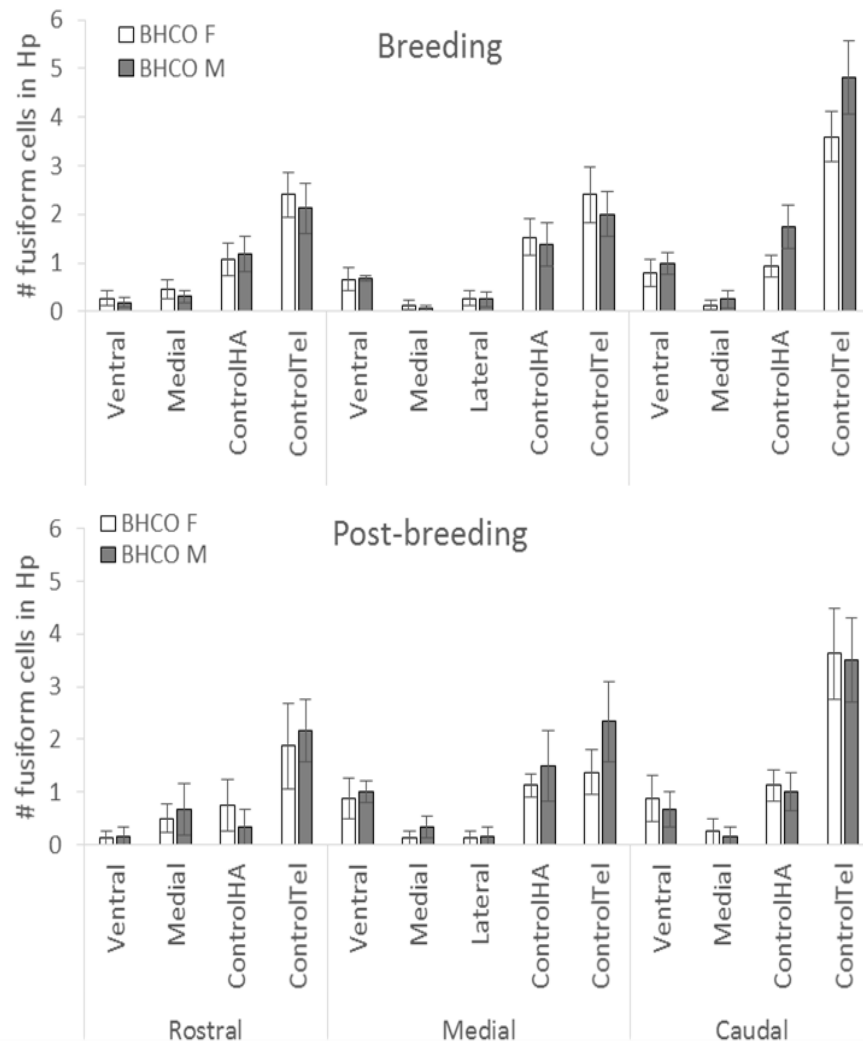


Figure 4.10

Figure 4.11. Mean number of fusiform cells \pm SE per field of view in the subventricular zone (SVZ), with the hippocampus (Hp) edge acting as the dependent variable and the telencephalon (Tel) edge acting as the covariate in the rostral, medial, and caudal regions of the brains of female and male brown-headed cowbirds and red-winged blackbirds in breeding and post-breeding conditions.

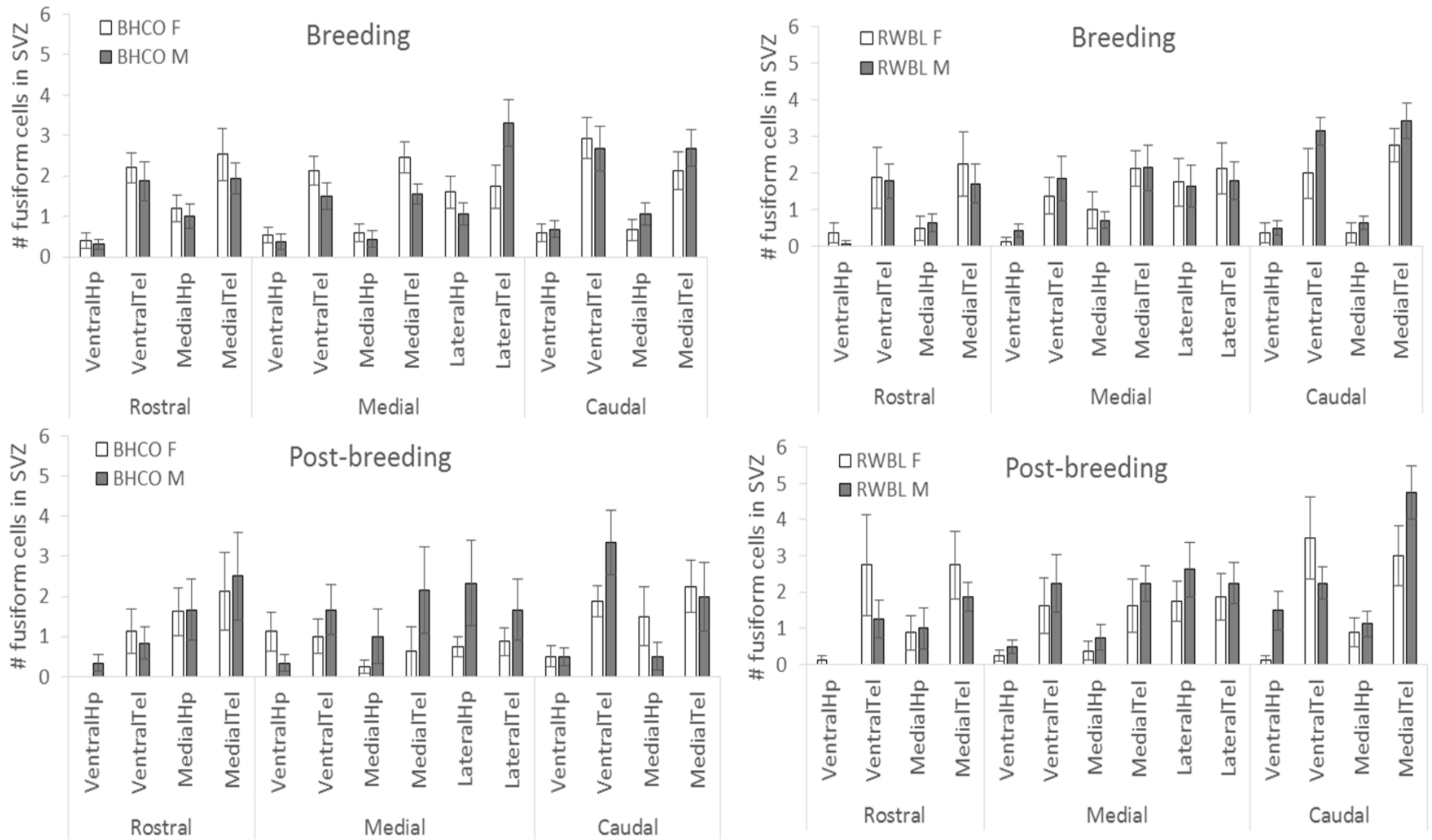


Figure 4.11

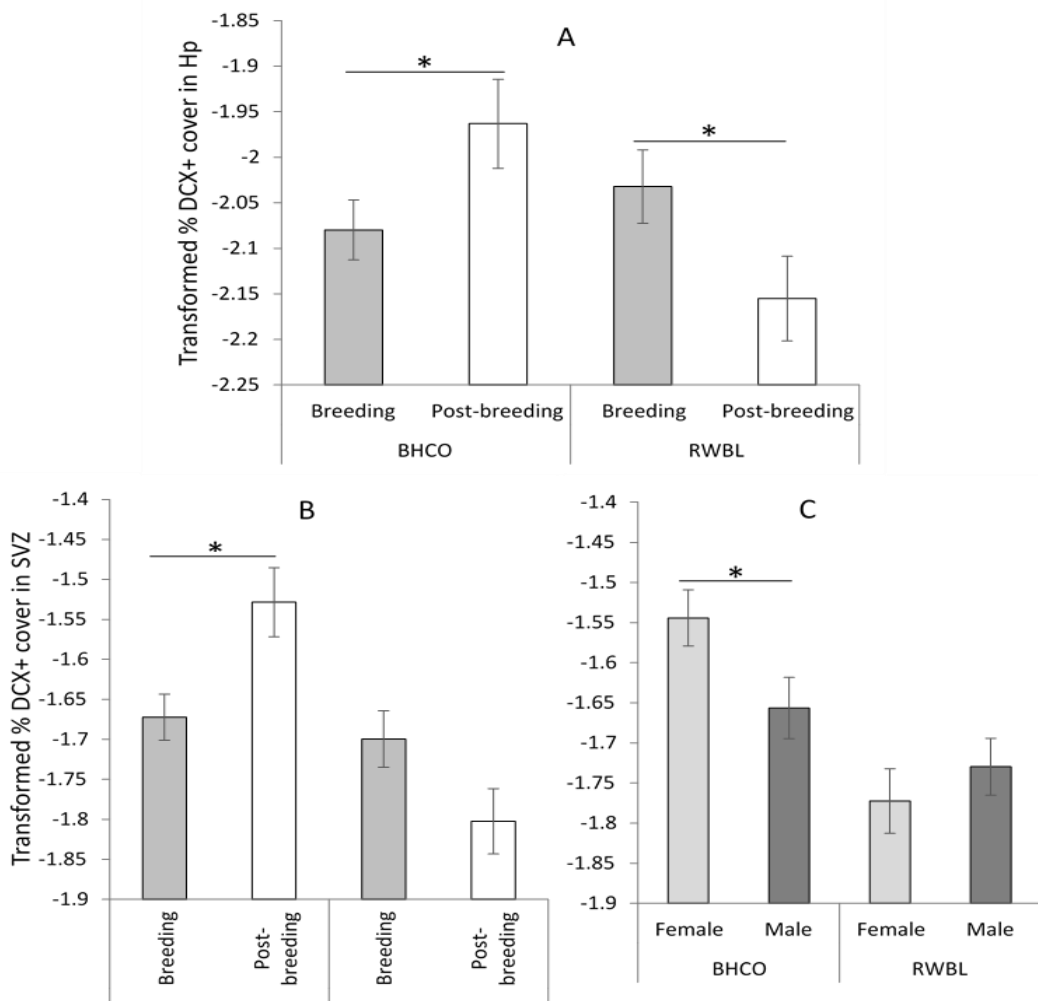


Figure 4.12 Significant interactions between sex (female and male), species (brown-headed cowbird and red-winged blackbird), and breeding condition (breeding and post-breeding) for % doublecortin immunoreactive (DCX+) cover inside the hippocampus (A) and on the hippocampus edge of the subventricular zone (SVZ) (B and C). Data presented are least square means adjusted to the covariate \pm SE. The covariate for (A) was the field of view in the telencephalon in the respective brain region whereas the covariate for B and C was the respective telencephalon edge for each SVZ field of view. The proportions of DCX+ cover were log arcsine square root transformed for analyses and for the figure. Asterisks indicate $p \leq 0.05$.

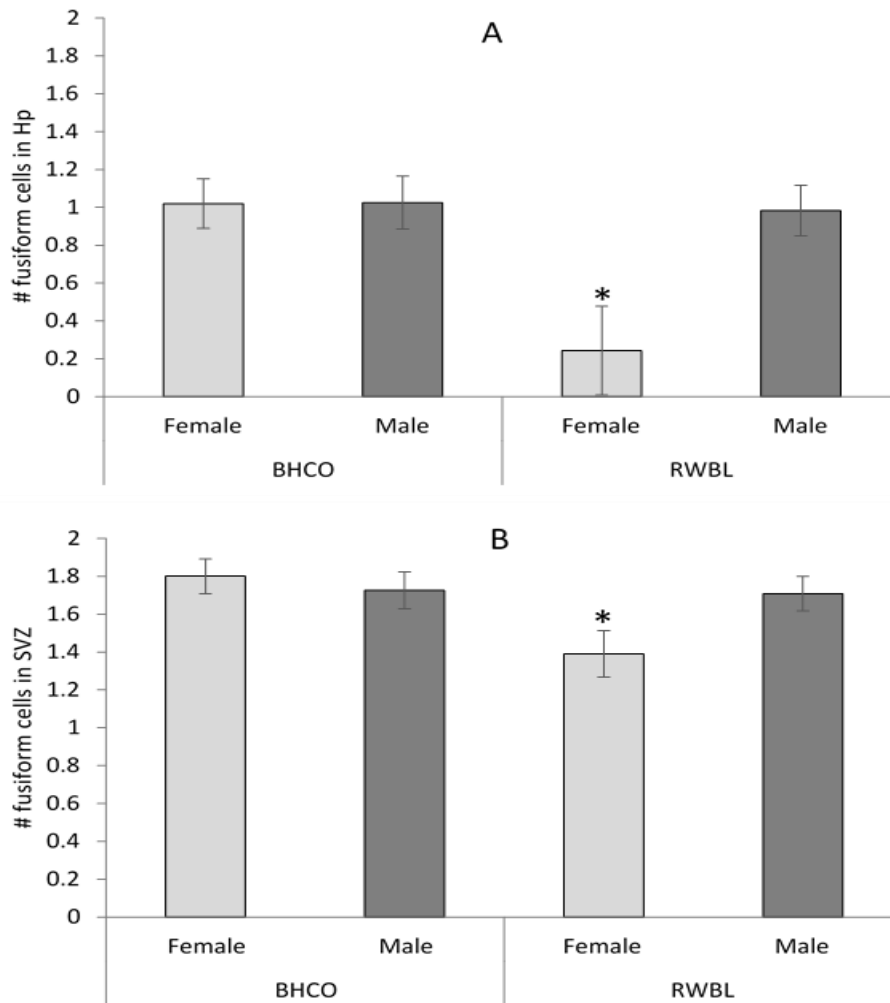


Figure 4.13 Significant interactions between sex (female and male) and species (brown-headed cowbird and red-winged blackbird) for the number of fusiform cells per field of view inside the hippocampus (A) and on the hippocampus edge of the subventricular zone (SVZ) (B). Data presented are least square means adjusted to the covariate \pm SE. The covariate for (A) was the field of view in the telencephalon in the respective brain region whereas the covariate for B was the respective telencephalon edge for each SVZ field of view. Asterisks indicate that female red-winged blackbirds have significantly fewer cells per field of view than any other group ($p \leq 0.05$).

interaction, with males having higher immunoreactivity than females in breeding condition in the medial field of view of the rostral section ($t_{449} = 2.18, p = 0.03$), whereas no sex difference existed in post-breeding condition ($t_{449} = 0.89, p = 0.37$; Table 4.4, Figure 4.6).

In the SVZ, there were multiple significant effects (Table 4.5). The following main effects were statistically significant: 1) species, with cowbirds having higher immunoreactivity than blackbirds and 2) area, with the ventral field of view in the caudal section having the highest immunoreactivity of all (Table 4.5, Figure 4.7). The following two-way interactions were statistically significant: 1) species by sex, with female cowbirds having more immunoreactivity than male cowbirds ($t_{75} = 2.17, p = 0.03$), but there was no sex difference in blackbirds ($t_{75} = 0.80, p = 0.43$), 2) species by breeding condition, with immunoreactivity being higher in post-breeding condition in cowbirds ($t_{75} = 2.77, p = 0.007$), but nearly significantly higher in breeding condition in blackbirds ($t_{75} = 1.89, p = 0.06$), 3) species by area, with cowbirds having more immunoreactivity than blackbirds in the medial field of view in the rostral section ($t_{449} = 4.36, p < 0.0001$) and the ventral field of view in the medial section ($t_{449} = 3.35, p = 0.0009$) (Table 4.5, Figures 4.7, 4.12). All other interactions were not significant (Table 4.5, Figure 4.7).

Round cells

Inside the hippocampus, area was the only statistically significant factor, with the ventral fields of view in the medial and caudal sections having the highest number of cells (Table 4.6, Figure 4.8).

In the SVZ, area was statistically significant, with the medial fields of view in the medial and caudal sections having the highest number of cells (Table 4.7, Figure 4.9).

Species by area was also statistically significant, with cowbirds having more cells per field of view than blackbirds in the medial field of view in the rostral section ($t_{449} = 2.16$, $p = 0.03$), and in the ventral field of view in the medial section ($t_{449} = 2.07$, $p = 0.04$) (Table 4.7, Figure 4.9).

Fusiform cells

Inside the hippocampus, there was a significant effect of species, with cowbirds having more cells per field of view than blackbirds, and a significant effect of sex, with males having more cells per field of view than females (Table 4.8, Figures 4.10, 4.13). There was a significant sex by species interaction, with female blackbirds having significantly fewer cells per field of view than male blackbirds ($z = 2.72$, $p = 0.006$), but there was no sex difference in cowbirds ($z = 0.03$, $p = 0.98$) (Figure 4.13). Female blackbirds drove the main effects of sex and species as this group also had a lower number of cells per field of view than male cowbirds ($z = 2.85$, $p = 0.004$) and female cowbirds ($z = 2.88$, $p = 0.004$) (Figure 4.13).

In the SVZ, there was a significant main effect of species, with cowbirds having more fusiform cells than blackbirds, and a significant effect of breeding condition, with more fusiform cells in post-breeding birds than in breeding birds (Table 3.9, Figure 4.11). There was also a significant sex by species interaction, with female blackbirds having fewer cells per field of view than male blackbirds ($z = 2.13$, $p = 0.03$), but there was no sex difference in cowbirds ($z = 0.56$, $p = 0.58$) (Figure 4.13). Female blackbirds also had fewer cells per field of view than female cowbirds ($z = 2.66$, $p = 0.008$) and male cowbirds ($z = 2.17$, $p = 0.03$) (Figure 4.13).

4.4 Discussion

Sex and seasonal patterns of hippocampus size and neurogenesis levels were consistent with my hypothesis that the hippocampus of cowbirds is specialized for brood parasitism. Cowbirds had a larger hippocampus, relative to the telencephalon, than blackbirds, and females had a larger hippocampus than males, with female cowbirds having the largest hippocampus of all groups tested (Table 4.3, Figures 4.4 and 4.5). Consistent with these hippocampal volume results, DCX immunoreactivity in the SVZ was higher in cowbirds than blackbirds and higher in female cowbirds than in male cowbirds with no sex difference in blackbirds (Table 4.5, Figures 4.7, 4.12). Cowbirds had higher % DCX+ cover and more round (immature differentiating) cells than blackbirds in some areas inside the hippocampus and in the SVZ (Tables 4.4, 4.5, 4.7 and Figures 4.6, 4.7, 4.9). With all areas pooled, cowbirds had more fusiform (migrating) cells than blackbirds inside the hippocampus and in the SVZ (Tables 4.8, 4.9, Figures 4.10, 4.11, 4.13). Although female blackbirds and cowbirds had a larger hippocampus than male blackbirds and cowbirds (Table 4.3, Figures 4.4, 4.5), female blackbirds had the fewest number of fusiform cells inside the hippocampus and in the SVZ of all groups (Tables 4.8, 4.9, Figures 4.10, 4.11). Unlike with hippocampus size, breeding condition had an effect on neurogenesis, with % DCX+ cover inside the hippocampus and in the SVZ, as well as the number of fusiform cells in the SVZ, being higher in post-breeding condition than breeding condition in cowbirds, but remaining stable or being higher in breeding condition in blackbirds (Tables 4.5, 4.6, 4.9, Figure 4.12, 4.13). In sum, patterns of hippocampus size and neurogenesis were similar, except for seasonal effects.

4.4.1 Volume

Cowbirds had a larger hippocampus than blackbirds, just like food-storers have a larger hippocampus than non-food-storers. The hippocampus in birds, as well as in mammals, is involved in spatial memory (reviewed by Colombo and Broadbent 2000). This association between the hippocampus and spatial memory has led to the hypothesis that enhanced spatial memory is accompanied by an evolutionary enlargement of the hippocampus relative to the size of the telencephalon in food-storing birds (Krebs et al. 1989, Sherry et al. 1989, Brodin and Lundborg 2003). In a meta-analysis of distantly related bird species, including brown-headed cowbirds and red-winged blackbirds, the relative volume of the hippocampus was positively related to the degree of food-storing specialization, after controlling for brood parasitism (Garamszegi and Eens 2004). Food storing places selection on both females and males for enhanced spatial memory, whereas brood parasitism, in which only females search for host nests, places selection on only half the population, therefore I would expect the effect of food storing to be stronger than brood parasitism. However, the larger hippocampus size, relative to telencephalon size, of cowbirds compared with blackbirds, suggests that selection on only one sex can generate species differences present in both sexes (Figure 4.5). Female and male cowbirds share all genes except the sex chromosomes and this genetic correlation can cause a trait favoured in one sex to occur in both (Lande 1980, Wyman et al. 2013). Genetic correlation may explain why possible sex-specific selection on female cowbirds has resulted in species differences in hippocampus size between brood-parasitic and non-brood-parasitic species, like species differences that exist between food-storers and non-food-storers.

In addition to a significant difference between species, I also found a significant effect of sex, with female blackbirds and cowbirds having a larger hippocampus than male blackbirds and cowbirds (Table 4.3, Figure 4.5). In contrast, Sherry et al. (1993) found that female cowbirds had a larger hippocampus than male cowbirds but no significant sex differences was detected in red-winged blackbirds and common grackles (*Quiscalus quiscula*), another icterid. My sample size was larger than the earlier study ($n = 32$ total subjects spread over three species in Sherry et al. 1993 versus $n = 87$ spread over two species in the current study) and Sherry et al. (1993) analyzed cowbirds and blackbirds separately, so similar results (i.e., main effects of species and sex) may have been reported with a larger dataset and if all data were analyzed simultaneously. Furthermore, the telencephalon was much larger in male blackbirds than in female blackbirds in my study, whereas telencephalon size was slightly (but not significantly) smaller in males in the earlier study (Sherry et al. 1993). Ecological or methodological differences may account for some of the differences between my study and the earlier study; however, given my larger sample size, I am confident that my results are representative of conditions within my own study site and year.

Why did I observe an overall sex difference as opposed to only observing a sex difference in cowbirds, as I had originally predicted? Cowbirds have only been parasites for about half the time than older parasites, such as cuckoos in Europe and Asia (Rothstein et al. 2002) and many life-history characteristics long believed to be brood parasitic adaptations (e.g.: shorter incubation period and faster growth rate) were actually inherited from a nonparasitic ancestor (Mermoz and Ornelas 2004). Thus, cowbirds likely have few specific adaptations to brood parasitism and their ancestor (and extant closely-

related species) likely already possessed traits that facilitated brood parasitism. For instance, perhaps the female nonparasitic ancestor of the cowbirds had a larger hippocampus than the male, a pre-adaptation to brood parasitism, and once cowbirds became brood parasites, selection on females to remember the location of host nests resulted in both sexes of cowbirds having a larger hippocampus than blackbirds. In addition to a female-biased sex difference in hippocampus size, red-winged blackbirds display other traits that resemble cowbirds, such as exaggerated begging behaviour (Rivers et al. 2013) and greater auditory sensitivity in females, which is not present in any other passerine (Gall et al. 2011); the icterid family may therefore be pre-adapted for brood parasitism (Mermoz and Ornelas 2004).

However, sex patterns for hippocampus size are not consistently female-biased in icterids. Specifically, red-winged blackbirds (current study), brown-headed cowbirds, and shiny cowbirds had a female-biased sex difference in relative hippocampus size while no sex difference was reported in common grackles, red-winged blackbirds (Sherry et al. 1993), bay-winged cowbirds and screaming cowbirds (Sherry et al. 1993, Robereda et al. 1996, Clayton et al. 1997). Nonetheless, breeding screaming cowbird females tended to have a larger relative hippocampus size than males. The sample size was only 12 individuals and so the study may have lacked the statistical power to detect a difference (Robereda et al. 1996; Clayton et al. 1997). To provide conclusive support that hippocampus size is female-biased generally in icterids, clearly more data are required from a larger number of icterid species, especially with large sample sizes for each species repeated across years and seasons.

No seasonal effects were present in hippocampus size, but they were present in whole telencephalon size. Unlike shiny and screaming cowbirds (Clayton et al. 1997), brown-headed cowbirds did not have a larger relative hippocampus in the breeding season (Table 4.3, Figure 4.4). However, the absence of a seasonal effect on relative hippocampal volume is consistent with brown-headed cowbirds performing equally well in non-breeding and breeding conditions on a large-scale spatial task (Chapter 2, Guigueno et al. 2014). Although there was no seasonal effect on hippocampus size relative to the telencephalon, the telencephalon itself was smaller in post-breeding condition (Table 4.3, Figure 4.4). Similar results in birds were reported in a meta-analysis by Smulders (2002) and more recently by De Groof et al. (2009) using magnetic resonance imaging with repeated measures. Changes in telencephalon volume could be caused by mechanisms that are not mutually exclusive, such as changes in neuropil, cell bodies and/or extracellular space (De Groof et al., 2009). Cell numbers could also change seasonally, although there is little support for this possibility. Functionally, decreasing brain matter in post-breeding condition could reduce the costs of maintaining energetically expensive brain tissue at a time when food is scarce (Yaskin 1984, Smulders 2002). In sum, regarding volume, brown-headed cowbirds have a seasonally stable hippocampus that is larger than the hippocampus of a non-parasitic relative, but, like previous studies, the whole telencephalon decreases in size in post-breeding condition.

4.4.2 Neurogenesis

DCX is a novel but reliable endogenous marker of neurogenesis (Balthazart and Ball 2014a, b). Few studies have simultaneously examined sex and seasonal changes in DCX+ in the hippocampus of any organism. In the current study, I assessed neurogenesis levels by quantifying DCX+ cells and fibres in fields of view inside the hippocampus and in the SVZ. Therefore, I could only compare the density of DCX+ cells and fibres per field of view between groups and not the total number of cells in the hippocampus.

Birds that rely more on spatial memory to reproduce and/or survive may have more hippocampal neurogenesis, especially if they need to encode new information about their surroundings (Barnea and Pravosudov 2011). In fact, food-storing black-capped chickadees have greater hippocampal neurogenesis, as measured by the exogenous marker BrdU, than non-food-storing house sparrows (Hoshooley and Sherry 2007). In addition, black-capped chickadees from harsher climates that rely more heavily on cached food have higher hippocampal neurogenesis than chickadees from milder climates (Chancellor et al. 2011). Finally, neurogenesis may be crucial for migrating birds that have been shown to have a larger hippocampus with more neurons than non-migrating birds (Pravosudov et al. 2006). The mechanism underpinning these species differences in hippocampus size and neuron number is likely increased neurogenesis, which is higher in migratory white-crowned sparrows than the non-migratory sub-species (LaDage et al. 2011). Migratory and food-storing birds may need to be equipped with a hippocampus that can accommodate heavier memory loads than non-migratory and non-food-storing birds (Barnea and Pravosudov 2011).

Brood parasites, like food-storing and migratory birds, may need a brain that can handle a heavier memory load. Thus, I predicted that cowbirds would have more neurogenesis than blackbirds. Indeed, relative to blackbirds, cowbirds had 1) higher %DCX+ cover in the medial region inside the hippocampus (Table 4.4, Figure 4.6), 2) higher %DCX+ cover in the SVZ overall, but specifically in the medial and rostral regions (Table 4.5, Figure 4.7) , 3) more differentiating immature neurons in the SVZ in the medial and rostral regions (Table 4.7, Figure 4.9), and 4) more migrating neurons both inside the hippocampus (Table 4.8, Figure 4.13A) and in the SVZ (Table 4.9, Figure 4.13B). The patterns of %DCX+ cover in the SVZ were similar to the volume results, as cowbirds had higher %DCX+ cover than blackbirds. In addition, female cowbirds showed more hippocampal neurogenesis than male cowbirds, with no sex difference in blackbirds (Tables 4.3, 4.5). This significant sex by species interaction provides the strongest support for the hypothesis that the hippocampus of female cowbirds is specialized for brood parasitism (Figure 4.12). In sum, female cowbirds, the group in my experimental design with the highest predicted memory load, had the most hippocampal neurogenesis.

Although hippocampal neurogenesis was most pronounced in female cowbirds, it was either similar between female and male blackbirds (%DCX+ cover) or least pronounced in female blackbirds (migrating neurons). Inside the hippocampus and in the SVZ, female blackbirds had fewer migrating neurons per field of view than male blackbirds, female cowbirds, and male cowbirds (Tables 4.8, 4.9, Figures 4.10, 4.11, 4.13). Therefore, although female blackbirds and cowbirds had a larger hippocampus, relative to the telencephalon, than male blackbirds and cowbirds, (Table 4.3) female

blackbirds and female cowbirds differed greatly in their levels of hippocampal neurogenesis. Perhaps the larger hippocampus size coupled with high levels of hippocampal neurogenesis are associated with the more accurate spatial memory needed for brood parasitism.

Hippocampal neurogenesis varied by breeding condition (Tables 4.4, 4.5, 4.9), even though hippocampal volume did not (Table 4.3). Neurogenesis was generally higher in post-breeding condition in cowbirds, whereas it was either stable or slightly higher in breeding condition in blackbirds (Figure 4.12, 4.13). In addition, neurogenesis in the rostral area inside the hippocampus was actually lower in females than in males in breeding condition (Table 4.4, Figure 4.6). These results seem counterintuitive as spatial memory load is expected to be highest in breeding condition, especially in females. However, as mentioned above, neurogenesis replaces old cells which may erase memories (Barnea and Pravosudov 2011); neurogenesis regulates forgetting in altricial and precocial rodents (Akers et al. 2014). It follows that cowbirds should replace old neurons at a time of the year when they no longer need to rely on old memories and replace them with new neurons required for new information to be encoded (Hoshooley and Sherry 2007, Barnea and Pravosudov 2011). Similarly, the song control nucleus HVC undergoes a peak in recruitment of new neurons in the autumn, a time at which some songbirds modify their song for the upcoming breeding season (Tramontin and Brenowitz 1999, Nottebohm 2004). Barnea and Nottebohm (1994) were the first to report seasonal variation in recruitment of new neurons into the hippocampus, however, further studies found no seasonal differences and Hoshooley and Sherry (2004) concluded that the results from Barnea and Nottebohm (1994) could only be explained by differences in

neuron survival. I did not measure the number of mature neurons in the hippocampus of cowbirds and blackbirds, therefore it is not known if neuron numbers changed seasonally. I can conclude, however, that hippocampal plasticity is greatest in the post-breeding condition, especially for female cowbirds that need to prepare their brains for a particularly high spatial memory load involving new information (i.e., the location of new host nests).

4.4.3 Summary and conclusions

Female brown-headed cowbirds are brood parasites that search for and parasitize host nests; male cowbirds do not search for nests. In contrast, no female-biased sex difference in spatial memory load exists in their nonparasitic relatives, red-winged blackbirds. I showed that the behaviour of cowbirds in the field is related to their neuroanatomy and neuronal plasticity.

Cowbirds had a larger hippocampus, relative to the telencephalon, than blackbirds, and females of both species had a larger hippocampus than males. Female cowbirds had the largest hippocampus of all four groups, but female blackbirds also had a larger hippocampus than males blackbirds. Stronger support for the specialization of the hippocampus for brood parasitism would have been a significant sex by species interaction, with female cowbirds having a larger hippocampus than male cowbirds and no sex difference in blackbirds.

Two alternative hypotheses for the patterns I observed in hippocampus size, beyond brood parasitism, are migratory patterns and differences in the telencephalon. Many bird species, including at least one species of icterid, have differential migratory

patterns whereby one sex migrates farther than the other. In the case of common grackles (*Quiscalus quiscula*), females tend to migrate farther than males (Dolbeer 1982). This pattern of longer migration in females may also exist in closely-related blackbirds and cowbirds. Furthermore, perhaps cowbirds have a more complex migration strategy that requires a larger hippocampus than blackbirds. Indeed, cowbirds tend to migrate further than blackbirds (Dolbeer 1982). However, I do not believe migration to be the reason behind the species and sex differences in hippocampus size that I reported in this study because shiny cowbirds, which are South American residents, also show female-biased sex difference in hippocampus size. In addition, brood-parasitic shiny and screaming cowbirds had a larger hippocampus than the non-parasitic bay-winged cowbird, which are all non-migratory (Reboreda et al. 1996). Alternatively, perhaps the trends are not driven by variation in hippocampus size but rather variation in telencephalon size (the covariate) such that males of both species have an ecological need for a larger telencephalon but similar needs for a hippocampus. However, I do not believe that to be the case as I know of no reason why blackbirds or males would require a larger relative telencephalon than cowbirds or females across breeding conditions, respectively. The song control nuclei, which are part of the telencephalon, are largest in the breeding season in males, so, I would have recorded a smaller hippocampus in breeding male blackbirds and cowbirds if the hippocampus trends were driven by the telencephalon size.

Patterns of neurogenesis into the hippocampus provided the strongest support for hypothesis that the hippocampus is specialized for a brood-parasitic lifestyle. In the SVZ, female cowbirds had higher %DCX+ coverage than males, whereas no sex difference existed in blackbirds. Hippocampal neurogenesis was highest in female cowbirds and

lowest in female blackbirds when counting migrating DCX+ cells per field of view, therefore female cowbirds had a combination of a large hippocampus and high hippocampal neurogenesis. Hippocampal neurogenesis was highest in post-breeding condition in cowbirds, which may ensure that old memories are purged at a time of the year when they are no longer necessary. In conclusion, patterns of hippocampal neurogenesis and hippocampus size among both sexes of two icterid species support the hypothesis that the brain of brood parasites is specialized for such behaviour in the wild.

4.5 References

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Chapter 5

5. Sex and seasonal differences in the volume and neurogenesis of song control nuclei in brown-headed cowbirds and red-winged blackbirds⁵

5.1 Introduction

The song control system (SCS) in the brain of songbirds is comprised of interconnected brain nuclei that control song production and learning. The SCS is comprised of two pathways: the caudal motor pathway and the anterior forebrain pathway. The caudal motor pathway, which is critical for song production, begins with the nucleus HVC, which sends projections to the robust nucleus of the arcopallium (RA), which in turn innervates the tracheosyringeal portion of the hypoglossal nucleus, followed by the syrinx, the song production organ (Nottebohm et al. 1976). The anterior

⁵ This chapter has not been published. David Sherry and Scott MacDougall-Shackleton will be co-authors when it is published as they were involved in the conceptual development, experimental design, and editing of the manuscript.

forebrain pathway, which is important for song learning and perception, also begins with HVC, but ends with RA, with three other nuclei along the pathway between HVC and RA (Scharff and Nottebohm 1991).

Two distinct and consistent observations regarding HVC and RA have been reported across studies and in a variety of species. First, males, who generally sing more than females, have a larger HVC and RA, and the sex differences in HVC and RA volumes correlate with the sex differences in the rates of singing and song complexity (MacDougall-Shackleton and Ball 1999, Hall et al. 2010). Second, HVC and RA increase drastically in size in breeding condition and are associated with increased singing rate and song stereotypy in some species (Tramontin and Brenowitz 2000). For example, the volume of HVC can be two to three times larger in breeding condition than in post-breeding condition (Smith 1996). Few studies have simultaneously examined sex and seasonal differences in the size of song control nuclei of at least two species differing in song development. Even fewer studies have also incorporated measures of neurogenesis.

An important mechanism behind seasonal changes in HVC volume is neurogenesis. Neuron numbers change seasonally with HVC volume in a variety of species (Tramontin et al. 2000, Smith et al. 1997, Smith et al. 1995, Tramontin et al. 1998). Song sparrows (*Melospiza melodia*), for example, have nearly double the number of neurons in HVC during the breeding season relative to the non-breeding season (Tramontin and Brenowitz 1999). As circulating testosterone and estrogen increase after the winter solstice, the survival of new neurons and the total number of neurons in HVC increase, whereas the rate of neurogenesis decreases (Rasika et al. 1994, Hidalgo et al. 1995, Tramontin and Brenowitz 1999). Neurogenesis is at its peak in the autumn when

there is a reduction in song stereotypy and song rate, although song can be functional at this time of year in many species (Kirn et al. 1994, Tramontin and Brenowitz 1999, 2000). Neurogenesis is not involved in seasonal RA volume increases because cell numbers in this nucleus do not change (Tramontin and Brenowitz 2000, Wada et al. 2014). Axons and dendrites in RA grow in breeding condition, along with changes in cell soma size and spacing (DeVoogd and Nottebohm 1981). In sum, there are seasonal changes in neuron number and neurogenesis in HVC, but none in RA.

Although a clear seasonal pattern of HVC neurogenesis has been reported across studies, few studies have examined sex differences in neurogenesis. Two studies reported sex differences in doublecortin immunoreactivity (DCX+; Balthazart et al. 2008, Hall and MacDougall-Shackleton 2012). Doublecortin (DCX) is a microtubule-associated protein that has recently been used as an endogenous marker of neurogenesis (Balthazart et al. 2008, Hall et al. 2012, Balthazart and Ball 2014a,b). With DCX, two immature cell types can be identified: fusiform cells, which are in the process of migrating to their final destination and round cells, which are immature differentiating neurons that have reached their final destination (Balthazart and Ball 2014a,b). In canaries (*Serinus canaria*) females have fewer fusiform cells than males (Balthazart et al. 2008), but in European starlings (*Sturnus vulgaris*) females have more fusiform and round cells than males (Hall and MacDougall-Shackleton 2012), even though females of both species sing less than males (reviewed in Hall et al. 2010). Thus, there is no clear pattern of sex differences in DCX+, as it varies according to species and likely also with season. Males in post-breeding condition sing less and have higher levels of neurogenesis than breeding males (see above) and male canaries that are housed with a female sing less and have more

DCX+ than males that are housed either alone or with another male (Balthazart et al. 2008, Alward et al. 2014). It seems that lower neurogenesis may be associated with less singing.

In the current study, I examined both sex and seasonal differences in HVC and RA volumes and DCX+ in HVC in brown-headed cowbirds (hereafter “cowbirds”; *Molothrus ater*) and red-winged blackbirds (hereafter “blackbirds”; *Agelaius phoeniceus*), two closely-related icterid species. Both species are open-ended learners (Marler et al. 1972, Yasukawa et al. 1980, King and West 1988, Brenowitz and Beecher 2005), but their song development and sexual differences in song differ. Cowbirds are obligate brood parasites, therefore cowbird nestlings are not exposed to a tutor of their own species. However, isolated males develop a normal song that is innately preferred by females (West et al. 1981). In the wild, young males likely develop their songs in winter roost flocks (King and West 1988). About 85% of yearling male songs are plastic between November to December, whereas only 20% of songs are plastic in February (King and West 1988). Male cowbirds modify their song in response to stimulation by females (King and West 1988, Hamilton et al. 1997). Male song likely plays a strong role in male fitness because females observed in the wild only mated with their partner after being courted by up to 14 males (Yokel 1986, Yokel and Rothstein 1991). Thus, female choice in cowbirds is likely a strong feature of such sexual selection. Female cowbirds do not sing at all (King and West 1990, Hamilton et al. 1997) whereas female blackbirds sing, although less than males (Nero 1956, Beletsky 1983, Kirn et al. 1989; reviewed by Hall et al. 2010). Male blackbirds that are acoustically isolated develop abnormal songs with some normal elements (Marler et al. 1972). New song types may be added to the

males' repertoires, which have likely evolved in response to male-male competition as opposed to female choice (Marler et al. 1972, Yasukawa et al. 1980).

The goal of this study was to investigate sex, species, and seasonal differences in the volumes of HVC and RA and neurogenesis in the HVC to determine whether patterns of volume and neurogenesis could reliably predict differences in song rate. Because males of both species sing more than females, especially during the breeding season, and that neurogenesis in the HVC is inversely related to signing rate in some birds, I predicted that males would have larger HVC and RA volumes with lower levels of HVC neurogenesis than females. In addition, I predicted that seasonal effects in volume and neurogenesis would be greater in males than in females, with larger HVC and RA volumes in breeding condition but more HVC neurogenesis in post-breeding condition. RA is primarily involved in the caudal motor pathway of the SCS, therefore I predicted that sex differences in RA size would be greater in cowbirds than in blackbirds because female cowbirds do not sing at all. Also, because female blackbirds sing, but female cowbirds do not, I predicted that female blackbirds would have larger or more discernable HVC than female cowbirds, especially in the breeding season, and that seasonal effects in HVC neurogenesis would be greater in female blackbirds. However, the HVC is involved in both the caudal motor pathway and the anterior forebrain pathway of the SCS and female song perception may be more important in cowbirds than in blackbirds. Therefore, it was also possible that no species differences in HVC volume and neurogenesis would exist between females, depending on the importance of song production versus perception in the HVC.

5.2 Methods

5.2.1 Subjects

I collected cowbirds and blackbirds of both sexes in breeding and post-breeding conditions (Table 5.1). Birds were the same as those used in Chapter 4. Birds from the breeding group were collected between mid-March and mid-May 2013 and birds from the post-breeding group were collected between mid-September and mid-November 2013. I captured all birds using ground traps and mist nets at various sites near Port Rowan, Ontario, Canada. Mean body weights \pm SE were as follows: 39.62 g \pm 0.63 (female cowbirds; $n = 22$), 50.33 g \pm 0.97 (male cowbirds; $n = 23$), 42.49 g \pm 0.66 (female blackbirds; $n = 16$), and 65.29 g \pm 0.98 (male blackbirds; $n = 23$). After capture, I transported the birds to the Advanced Facility for Avian Research at the University of Western Ontario in London, Ontario, where they were housed overnight in individual cages with food and water.

5.2.2 Blood sampling

I collected blood samples in the field to confirm breeding condition. I used the same birds as in Chapter 4, therefore I will refer to the table and figure summarizing androgen concentrations from that chapter. I took a blood sample from each bird by puncturing the brachial vein with a 26-gauge needle. Nearly all the samples were taken within 30 min, however 14 out of 88 samples were taken 30-92 minutes after capture. The correlation between androgen concentration and time was not statistically significant,

Table 5.1. Number of brains collected from each experimental group for the % discernable HVC, volume analyses measured by NeuN immunohistochemistry for HVC and RA, and neurogenesis (HVC only), measured by doublecortin immunohistochemistry. Brains were collected the day after the birds were captured from the field in breeding (March-May) and post-breeding (September-November) conditions.

Breeding condition	Brown-headed cowbird		Red-winged blackbird	
	Female	Male	Female	Male
HVC				
Discernability (total)				
Breeding	40% (15)	100% (16)	100% (8)	100% (15)
Post-breeding	25% (8)	88% (8)	38% (8)	88% (8)
HVC				
(Volume)				
Breeding	6	16	8	15
Post-breeding	2	7	3	7
HVC				
(Neurogenesis)				
Breeding	6	16	8	16
Post-breeding	2	6	3	7
RA, Telencephalon				
(Volume)				
Breeding	15	16	8	16
Post-breeding	8	8	8	8

therefore I kept these data points in the analyses. I collected approximately 400 μL of blood into heparinized capillary tubes and centrifuged the blood for 10 min at 13,000 \times g. Finally, I extracted the plasma from the tubes with a Hamilton syringe and froze the plasma at $-30\text{ }^{\circ}\text{C}$ until the hormone assay.

5.2.3 Androgen assay

Circulating androgen levels increase in breeding cowbirds (Dufty and Wingfield 1986 a,b, Guigueno et al. 2014, Chapters 2-3). I assayed plasma androgen concentration using a testosterone enzyme immunoassay previously validated for a variety of bird species (EIA; Cat. #1-2402, Salimetrics; [Washburn et al. 2007]). I previously validated the assay in cowbirds (Chapters 2-3, Guigueno et al. 2014; see also Newman et al. 2008) and I used the same protocol to validate the assay for blackbirds. The validation below is identical to the one in Chapter 4 because I used the same birds for both chapters. I assayed a serial dilution of blackbird plasma and compared measured levels of androgens in the dilutions to the standard curve using an ANCOVA. A non-significant interaction term ($F_{1,10} = 0.01$, $p = 0.94$) indicated that the slopes were similar and that the assay was suitable for blackbirds. Intra-assay variation was 8.85% and inter-plate variation, based on a pooled blackbird plasma sample and low and high controls was 3.79%. The sensitivity of the assay was 5 pg/mL (two standard deviations from the average value of zero on the standard curves), so undetectable levels were assigned a value of 2.5 pg/mL for the statistical analyses.

5.2.4 Brain collection

I deeply anesthetized the birds using isoflurane the day after capture. I transcardially perfused the birds with heparinized saline, followed by 4% paraformaldehyde. The brains were then carefully removed from the skull and placed in 4% paraformaldehyde for 24 h, followed by 30% sucrose for 48-72 h (until the brains sunk to the bottom of the vial). Finally, I froze the brains on powdered dry ice and stored them in aluminium foil at -80 °C until the start of immunohistochemistry.

5.2.5 Immunohistochemistry

I sectioned the brains into 40 µm sections in the coronal plane using a cryostat. Two sets of brain sections, each set two sections apart throughout the HVC and RA, were collected for NeuN and DCX immunohistochemistry. NeuN is a protein expressed in most mature neurons (Mullen et al. 1992) and was used to delineate HVC and RA to calculate their volume (Newman et al. 2008), whereas DCX is a protein expressed by migrating and immature differentiating neurons (Francis et al. 1999, Gleeson et al. 1999), and was used to quantify neurogenesis (Balthazart and Ball 2014a,b). Each run consisted of a randomly-selected sample of brains from different groups (Table 5.1). I ran NeuN and DCX immunohistochemistry using the protocols described in Chapter 4.

5.2.6 Microscopy

I used the NeuN-labelled sections to measure the volumes of HVC and RA. I captured images of HVC and RA with a Spot Idea 5-megapixel digital camera (Diagnostics Instruments) mounted on a Zeiss Axiophot microscope using a 1.25X

objective lens. Only a random bird ID was assigned to each photo, therefore the images were analyzed without reference to species, sex, or season. The perimeters of HVC and RA were traced in ImageJ software (NIH) (Figure 5.1). I summed the frusta volumes (truncated cone) between sections (80 μm) to estimate the total volumes of HVC and RA in both hemispheres. I used the same telencephalon measurements as those used in Chapter 4. HVC, RA, and telencephalon volumes used in the analyses for each bird were the average between hemispheres. I adjusted the sampling interval and used the next nearest section if a section was damaged or lost. In some groups HVC was indiscernible, especially in female cowbirds and in post-breeding female blackbirds. For one male breeding blackbird, tissue was too damaged to measure its HVC volume. One brain was damaged during the sectioning and could not be used for any volume measurement, hence the total sample size for RA and telencephalon volumes ($n = 87$) is one fewer than the total sample sizes for androgen measurements ($n = 88$). Final sample sizes for HVC, RA, and telencephalon volume according to experimental group are found in Table 5.1. I used DCX-labelled sections to quantify neurogenesis in HVC (Figure 5.2). I captured images of % DCX+ cover (cells and projections) and DCX+ round and fusiform cells with a Leica DFC 420C camera mounted to a Leica DM5500B microscope (Figure 5.3). I chose five sections 80 μm apart and centered in the larger part of the HVC from the hemisphere that was most intact and best stained. I analyzed two fields of view per section. One field of view was positioned in the centre of the HVC, whereas the other was positioned just outside and ventral to HVC (in the nidopallium; see Wada et al. 2014 for schematic drawing). I averaged results from all five sections for the analyses. I did not analyze DCX+ cells in RA because previous work has shown that there is little to

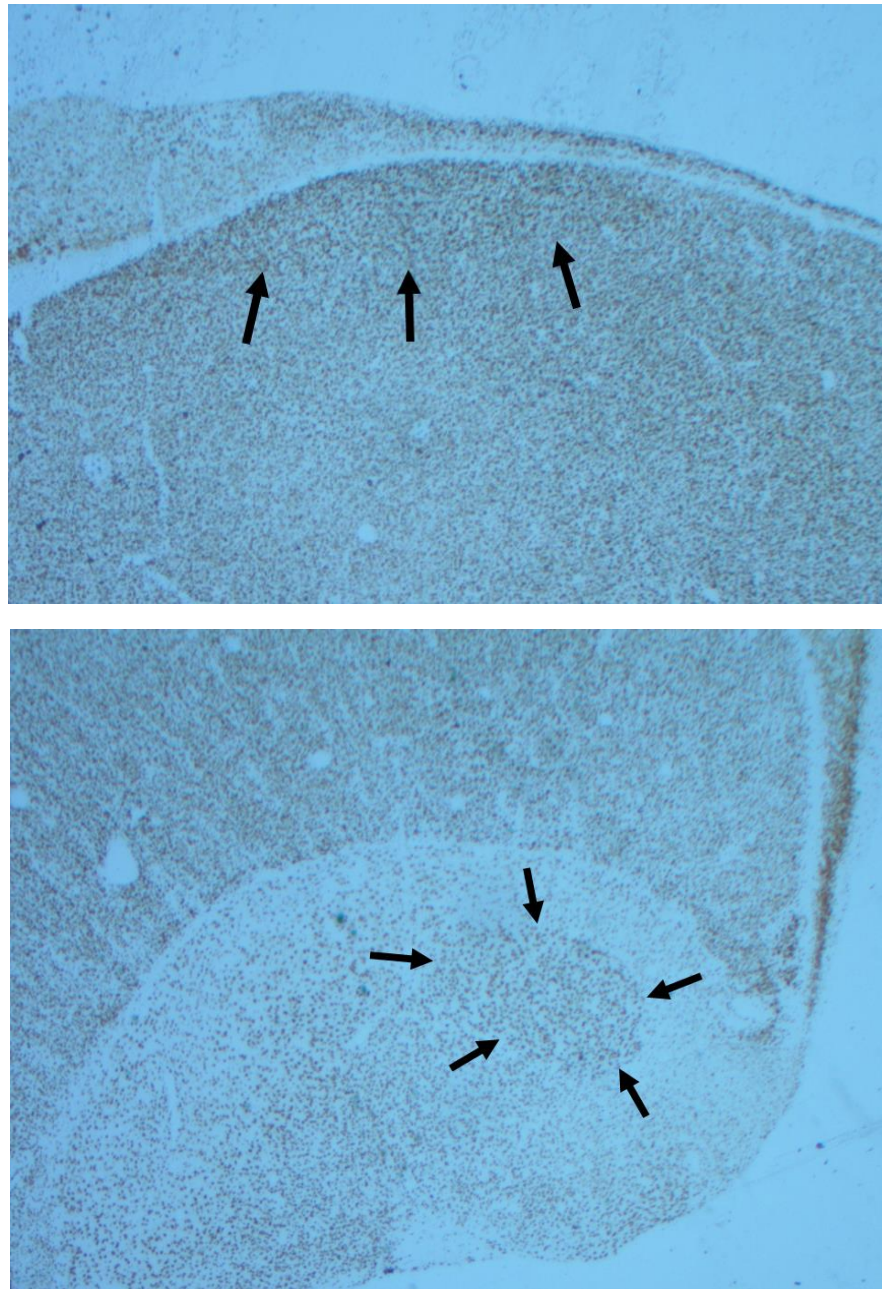


Figure 5.1 NeuN labeled brain sections with HVC (top) and robust nucleus of the arcopallium (RA) (below) indicated by arrows.

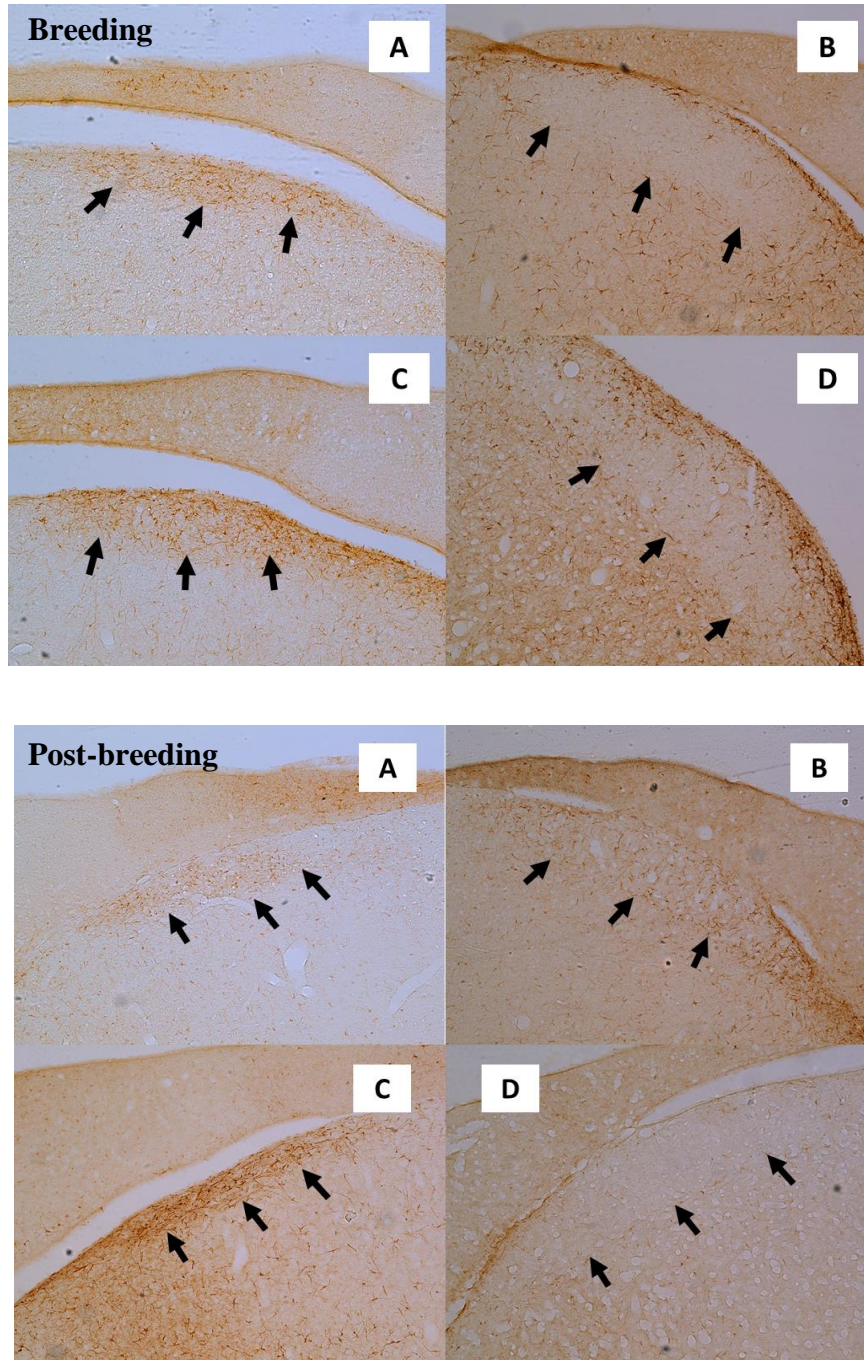


Figure 5.2 Doublecortin labelled brain sections with HVC indicated by arrows for breeding (top) and post-breeding conditions (bottom) in female (A) and male (B) brown-headed cowbirds (*Molothrus ater*) and female (C) and male (D) red-winged blackbirds (*Agelaius phoeniceus*).

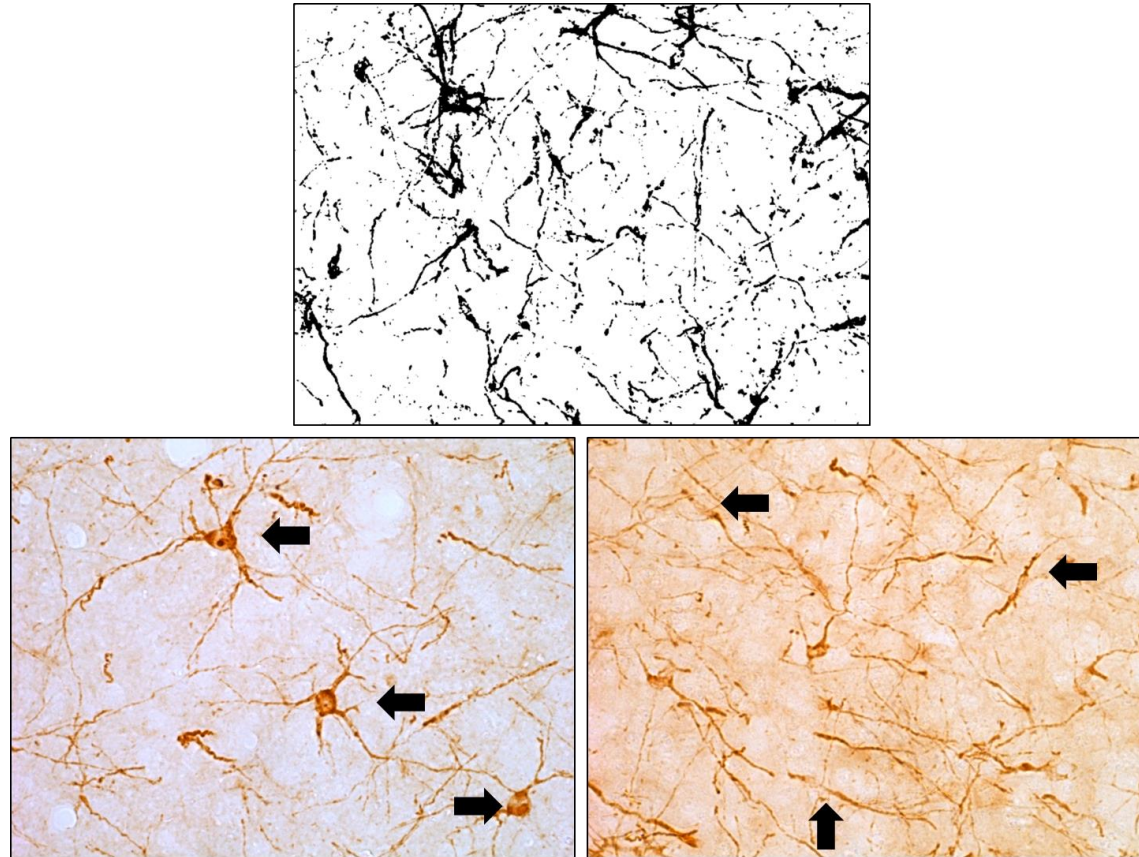


Figure 5.3 Fields of view in doublecortin-labeled sections, with an example of each type of measurement taken: thresholding to measure the % doublecortin immunoreactive cover (top), number of round cells, indicated by arrows (bottom left) and number of fusiform cells, indicated by arrows (bottom right)

no immunoreactivity in this song control nuclei in other passerines (Boseret al. 2007, Balthazart et al. 2008, Wada et al. 2014). For each field of view, I captured z-stack images in 0.63 μm steps through the focal planes with a 40X objective lens. Following Hall et al. (2010), I compiled these images using the montage mode in Leica Application Suite software, which resulted in an image that displayed all DCX+ cells and projections in focus. I used the threshold feature in ImageJ to calculate the % coverage by DCX+ cells and projections. I counted and analyzed fusiform cells and round cells separately. I was not able to quantify neurogenesis in some birds due to poor staining, therefore sample sizes from the DCX analyses differed from those for the volume analyses (see Table 5.1).

5.2.7 Data analysis

I conducted all statistical analyses in SAS (version 9.3, SAS Institute Inc., Cary, NC, USA). HVC was not always discernable in females (Table 5.1), therefore I ran Fisher exact tests to determine whether the proportion of females with discernable HVC changed between the breeding conditions for blackbirds and for cowbirds. For the volume analyses, I used general linear models (PROC GLM) with species, sex, breeding condition, and all interactions as explanatory variables, telencephalon volume (minus HVC or RA) as a covariate, and HVC and RA volumes as the dependent variables. I also tested whether telencephalon volume differed between species, sex, breeding condition with a general linear model (with all interactions). To analyze the average %DCX+ cover, number of round cells, and number of fusiform cells inside the HVC, I used general linear models, with species, sex, breeding condition, and all interactions as

explanatory variables, and the respective DCX+ measurements in the nidopallium as the covariate. To produce normally distributed residuals, I arcsine-transformed proportions from the %DCX+ cover data and log-transformed the remaining data if the residuals were not already normally distributed. Significant interactions were further analyzed using predetermined Fisher's LSD post-hoc tests. Data are presented as means \pm SE and results were considered significant if $p \leq 0.05$.

5.3 Results

5.3.1 Androgens

There was a significant main effect of breeding condition, with higher androgen levels in breeding condition than in post-breeding condition, and a significant main effect of sex, with males showing higher levels than females (same data as Chapter 4). Species differences and all interactions were not significant (Chapter 4).

5.3.2 Discernable HVC in females

A 4 X 2 Fisher exact test indicated there was a significant difference in the frequency of discernable HVC across breeding and post-breeding female blackbirds and cowbirds ($p = 0.008$; Table 5.1). I used 2 X 2 Fisher exact tests for pairwise comparisons. Breeding female blackbirds had more discernable HVC than breeding female cowbirds ($p = 0.007$; Table 5.1). Female blackbirds had more brains with a discernable HVC in breeding condition than in post-breeding condition ($p = 0.03$; Table 5.1). Finally, in female cowbirds, there was no significant difference in the proportion brains with a

discernable HVC between breeding condition and post-breeding condition ($p = 0.66$; Table 5.1). It was only possible to include data from the birds with a discernable HVC for the volume and neurogenesis analyses below (Table 5.1).

5.3.3 Volume

Males had a larger HVC than females and the relative HVC volume was larger in breeding birds than in post-breeding birds, whereas there was no main effect of species (Table 5.2, Figure 5.4A). There was a significant sex by breeding condition interaction, with males having a larger HVC in breeding condition than in post-breeding condition ($t_{55} = 4.53, p < 0.0001$), with no seasonal change in females ($t_{55} = 0.27, p = 0.78$) (Table 5.2, Figure 5.4A). All other interactions were not significant (Table 5.2)

Males had a larger relative RA volume than females and RA was larger in breeding birds than in post-breeding birds, whereas there was no main effect of species (Table 5.2, Figure 5.4B). There was a significant sex by breeding condition interaction, with males having a larger RA in breeding condition than in post-breeding condition ($t_{78} = 5.08, p < 0.0001$), with no seasonal change in females ($t_{78} = 1.84, p = 0.07$) (Table 5.2, Figure 5.4B). There was a significant species by sex interaction, with the sex difference in cowbirds ($t_{78} = 23.15, p < 0.0001$) being greater than the sex difference in blackbirds ($t_{78} = 14.25, p < 0.0001$) (Table 5.2, Figure 5.4B). Finally, there was a significant species by breeding condition interaction with a greater effect of breeding condition in blackbirds ($t_{78} = 4.81, p < 0.0001$), than in cowbirds ($t_{78} = 2.00, p = 0.05$) (Table 5.2, Figure 5.4B). The three-way interaction between species, sex, and breeding condition was not statistically significant (Table 5.2).

Table 5.2. Summary of statistical effects of species, sex, breeding condition (BC) and their interactions on the volumes of the HVC, RA, and the telencephalon. Results are from a general linear model. Significant effects are in bold.

Factors	<i>F</i>	d.f.	<i>p</i> -value
HVC volume			
Species	0.71	1,55	0.40
Sex	42.36	1,55	<0.0001
BC	5.08	1,55	0.03
Species x Sex	0.15	1,55	0.70
Species x BC	1.85	1,55	0.18
Sex x BC	8.23	1,55	0.006
Species x Sex x BC	0.03	1,55	0.86
Telencephalon (covariate)	15.25	1,55	0.0003
RA volume			
Species	0.27	1,78	0.61
Sex	603.67	1,78	<0.0001
BC	21.20	1,78	<0.0001
Species x Sex	16.26	1,78	0.0001
Species x BC	5.13	1,78	0.03
Sex x BC	5.65	1,78	0.02
Species x Sex x BC	1.78	1,78	0.19
Telencephalon (covariate)	22.38	1,78	<0.0001
Telencephalon volume			
Species	96.82	1,79	<0.0001
Sex	24.40	1,79	<0.0001
BC	21.82	1,79	<0.0001
Species x Sex	5.53	1,79	0.02
Species x BC	0.01	1,79	0.90
Sex x BC	0.89	1,79	0.35
Species x Sex x BC	0.45	1,79	0.51

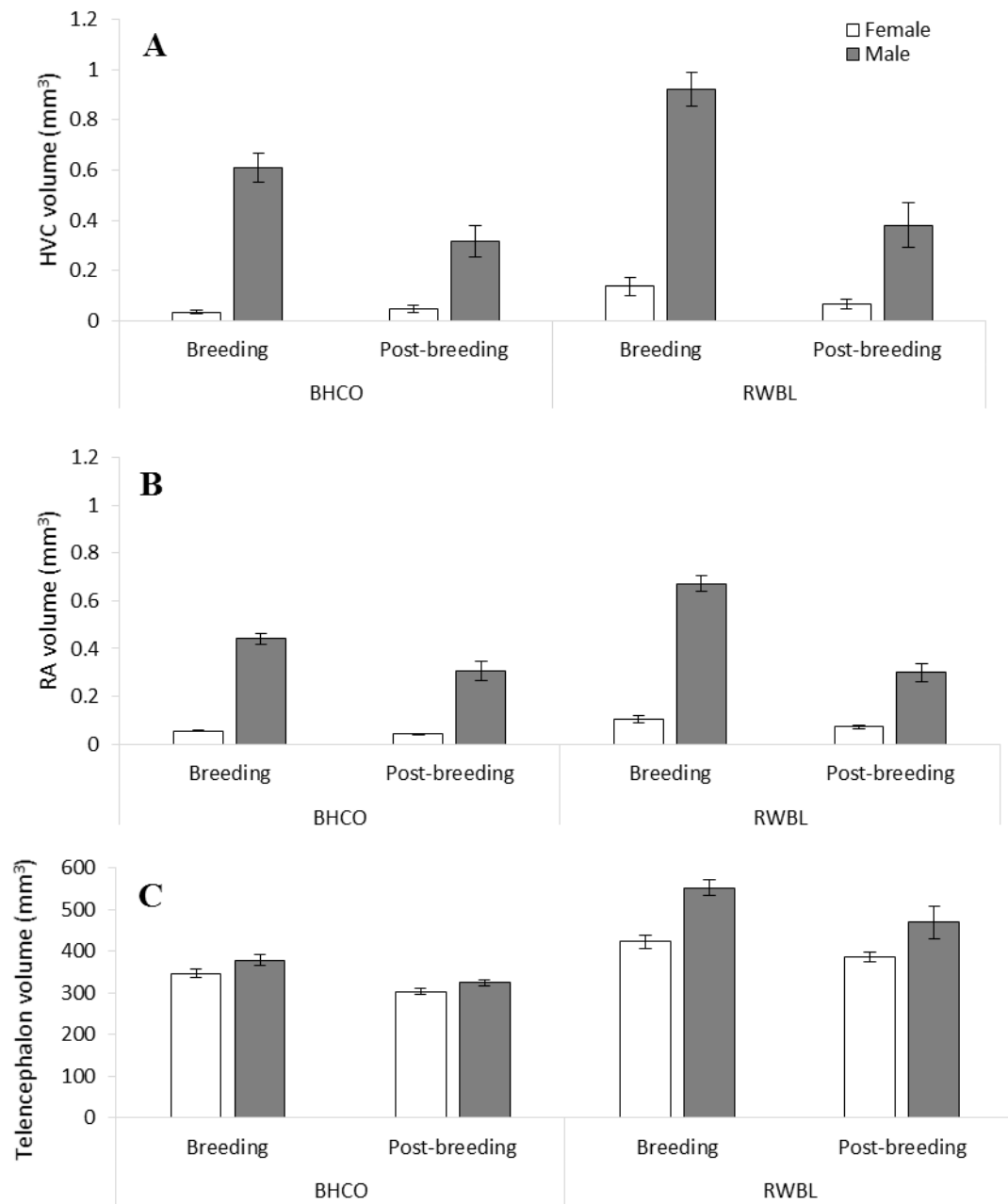


Figure 5.4. Mean \pm SE volumes of HVC, RA, and telencephalon in female and male brown-headed cowbirds (BHCO) and red-winged blackbirds (RWBL) in breeding and post-breeding conditions. Overall, males had larger HVC and RA relative to telencephalon volume than females.

For the telencephalon, there was a significant effect of species, with blackbirds having a larger telencephalon than cowbirds (Table 5.2, Figure 5.4C). There were also significant effects of sex, with males having a larger telencephalon than females, and of breeding condition, with breeding birds having a larger telencephalon than post-breeding birds (Table 5.2, Figure 5.4C). There was a significant species by sex interaction, with male blackbirds having a larger telencephalon than female blackbirds ($t_{79} = 4.98$, $p < 0.0001$), whereas there was only a nearly significant effect of sex in cowbirds ($t_{79} = 1.90$, $p = 0.06$) (Table 5.2, Figure 5.4C).

5.3.4 Doublecortin

In HVC, females had higher %DCX+ levels than males, whereas there were no significant effects of species and breeding condition (Table 5.3, Figure 5.5). There was a significant sex by breeding condition interaction, with %DCX+ levels being higher in males in post-breeding condition in males ($t_{55} = 4.14$, $p = 0.0001$), but higher in females in breeding condition ($t_{55} = 1.46$, $p = 0.02$) (Table 5.3, Figure 5.5). All other interactions were not significant (Table 5.3).

Females had more round cells than males per field of view, whereas there were no main effects of species or breeding condition (Table 5.3, Figure 5.6). There was a significant sex by breeding condition interaction, with males having more round cells per field of view in post-breeding condition than in breeding condition ($t_{55} = 3.56$, $p = 0.0008$), whereas there was no seasonal effect in females ($t_{55} = 0.17$, $p = 0.86$) (Table 5.3, Figure 5.6). All other interactions were not significant (Table 5.3, Figure 5.6).

Table 5.3. Summary of statistical effects of species, sex, breeding condition (BC) and their interactions on the doublecortin immunoreactivity (DCX+) in the HVC of female and male brown-headed cowbirds and red-winged blackbirds in breeding and post-breeding conditions. DCX+ is a measure of neurogenesis. Results are from a general linear model. Significant effects are in bold.

Factors	<i>F</i>	d.f.	<i>p</i> -value
%DCX+ cover			
Species	3.76	1,55	0.06
Sex	71.40	1,55	<0.0001
BC	0.01	1,55	0.92
Species x Sex	0.51	1,55	0.48
Species x BC	0.60	1,55	0.44
Sex x BC	19.52	1,55	<0.0001
Species x Sex x BC	1.02	1,55	0.32
Telencephalon (covariate)	42.81	1,55	<0.0001
Round cells			
Species	0.71	1,55	0.40
Sex	30.62	1,55	<0.0001
BC	3.08	1,55	0.08
Species x Sex	3.32	1,55	0.07
Species x BC	2.37	1,55	0.13
Sex x BC	4.13	1,55	0.05
Species x Sex x BC	0.08	1,55	0.78
Telencephalon (covariate)	11.17	1,55	0.002
Fusiform cells			
Species	0.03	1,55	0.86
Sex	5.10	1,55	0.03
BC	1.33	1,55	0.25
Species x Sex	10.89	1,55	0.002
Species x BC	4.64	1,55	0.04
Sex x BC	7.90	1,55	0.007
Species x Sex x BC	3.32	1,55	0.07
Telencephalon (covariate)	28.32	1,55	<0.0001

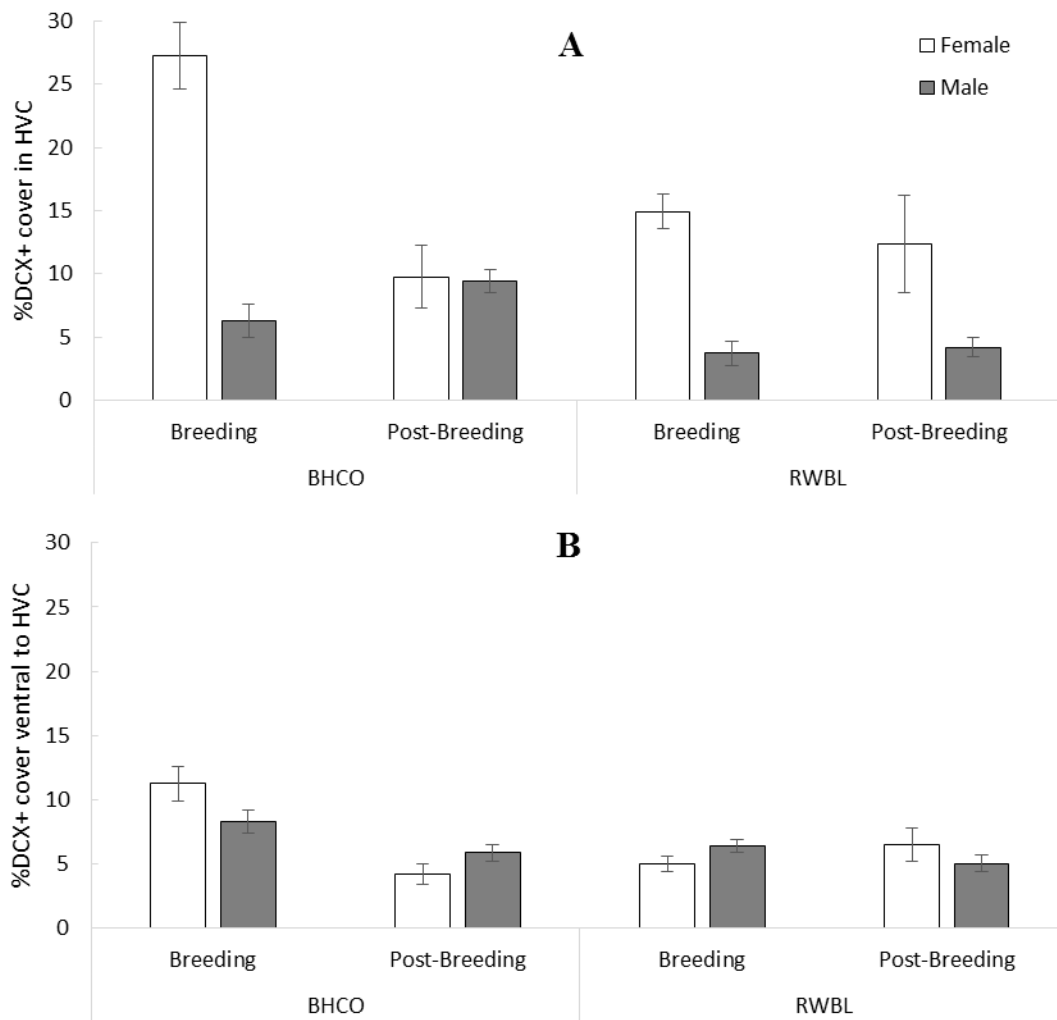


Figure 5.5. Mean \pm SE %DCX+ cover in fields of view inside (A) and outside (B) the HVC in female and male brown-headed cowbirds (BHCO) and red-winged blackbirds (RWBL) in breeding and post-breeding conditions. Means were calculated from five fields of view inside the HVC and five fields of view outside the HVC over five coronal sections centered in the middle of the HVC. Overall, females had higher levels of doublecortin immunoreactivity inside the HVC relative to outside the HVC than males.

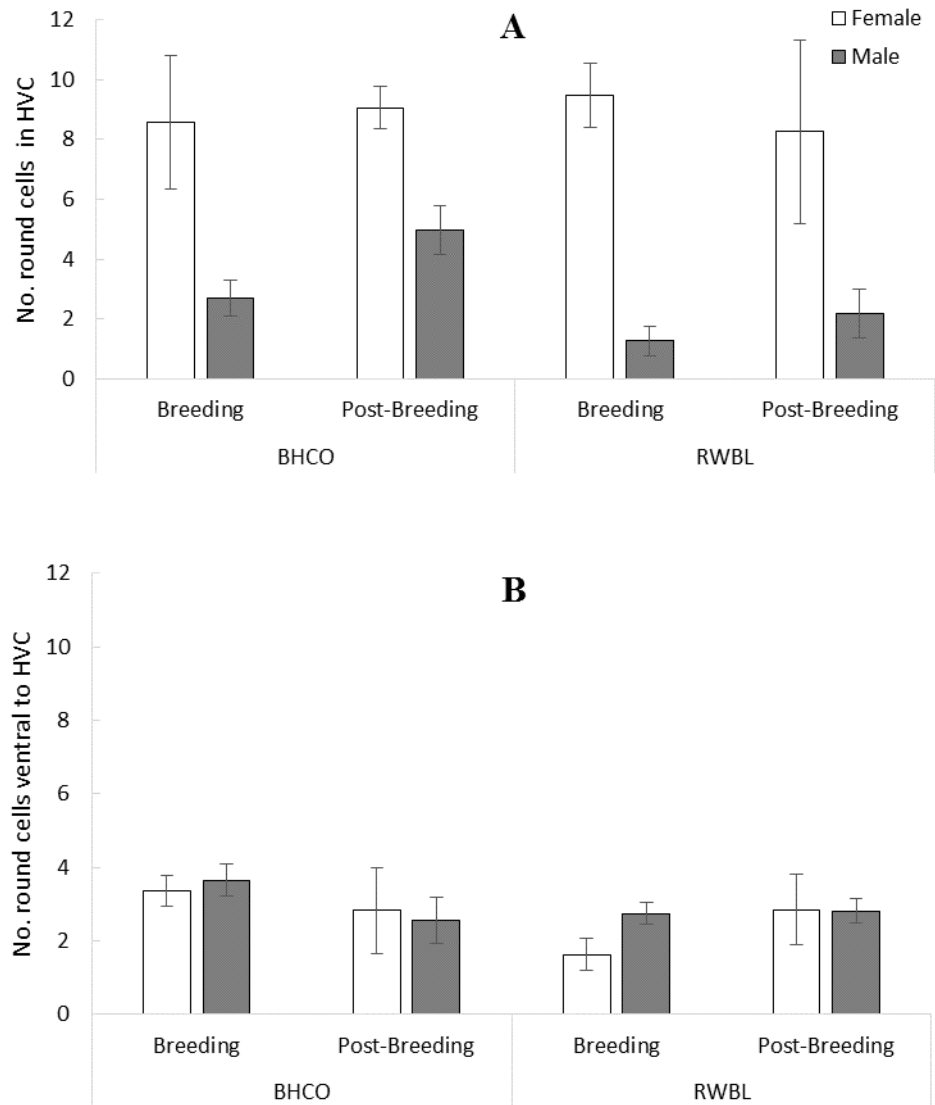


Figure 5.6. Mean \pm SE number of round cells per field of view inside (A) and outside (B) the HVC in female and male brown-headed cowbirds (BHCO) and red-winged blackbirds (RWBL) in breeding and post-breeding conditions. Means were calculated from five fields of view inside the HVC and five fields of view outside the HVC over five coronal sections centered in the middle of the HVC. Overall, females had more round cells per field of view inside the HVC relative to outside the HVC than males.

Females had more fusiform cells than males per field of view, whereas there were no effects of species and breeding condition (Table 5.3, Figure 5.7). There was a significant sex by breeding condition interaction, with males having more fusiform cells per field of view in post-breeding condition than in breeding condition ($t_{55} = 3.80$, $p = 0.0004$), whereas there was no seasonal effect in females ($t_{55} = 0.93$, $p = 0.36$) (Table 5.3, Figure 5.7). There was a significant species by sex interaction, with female blackbirds having more fusiform cells per field of view than female cowbirds ($t_{55} = 2.03$, $p = 0.05$), but male cowbirds having more fusiform cells than male blackbirds ($t_{55} = 2.98$, $p = 0.004$) (Table 5.3, Figure 5.7). Finally, there was a significant species by breeding condition interaction, with blackbirds having more fusiform cells per field of view in post-breeding condition than in breeding condition ($t_{55} = 2.70$, $p = 0.009$), whereas no seasonal effects exist in cowbirds ($t_{55} = 0.67$, $p = 0.51$) (Table 5.3, Figure 5.7). The three-way interaction was not significant (Table 5.3).

5.4 Discussion

I found multiple effects of sex, season, and species in the volume and neurogenesis analyses of song control nuclei. Female blackbirds, which sing more than female cowbirds, did not have a significantly larger HVC, but had more discernable HVC than female cowbirds, especially in breeding condition (Table 5.1). As predicted, males, which sing more than females, had larger HVC and RA than females and their HVC and RA were greater in volume in breeding condition, when rates of singing are highest (Table 5.2, Figures 5.4A, 5.4B). Consistent with my prediction, there was a greater sex difference in RA volume in cowbirds than in blackbirds, likely because female cowbirds

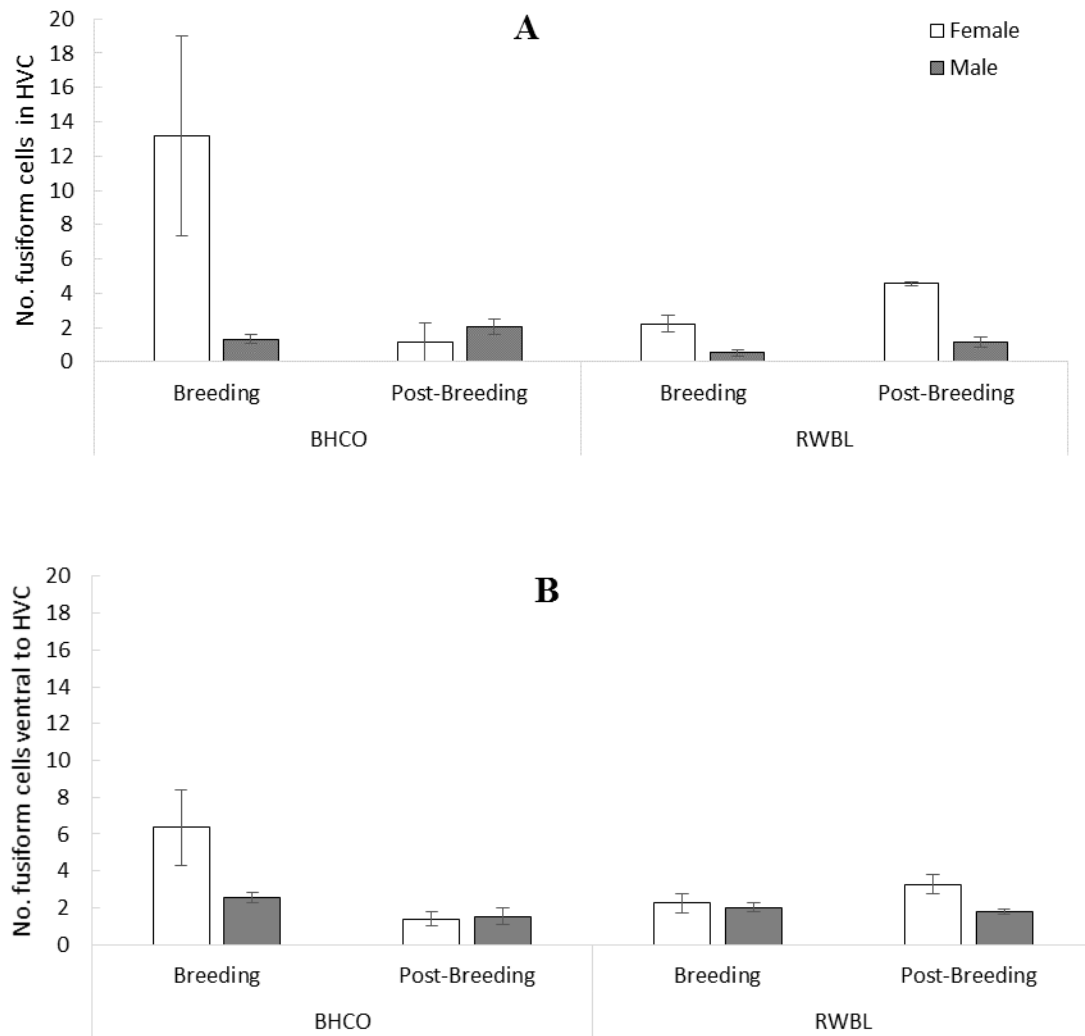


Figure 5.7. Mean \pm SE number of fusiform cells per field of view inside (A) and outside (B) the HVC in female and male brown-headed cowbirds (BHCO) and red-winged blackbirds (RWBL) in breeding and post-breeding conditions. Means were calculated from five fields of view inside the HVC and five fields of view outside the HVC over five coronal sections centered in the middle of the HVC. Overall, females had more fusiform cells per field of view inside the HVC relative to outside the HVC than males.

do not sing at all (Hamilton et al. 1997; Table 5.2, Figure 5.4B). Breeding condition had a greater influence on RA volume in blackbirds than in cowbirds, a seasonal effect that I had not predicted (Table 5.2, Figure 5.4B). Because female starlings had higher levels of neurogenesis in their HVC than males and that neurogenesis in the HVC of male canaries was inversely related with singing rate which varies according to breeding and housing conditions, I predicted that lower singing rate would be associated with higher neurogenesis. Indeed, females had higher neurogenesis, as indicated by the density of DCX+ cells and fibres, in HVC than males and males had higher neurogenesis levels in post-breeding condition (Table 5.3, Figures 5.5, 5.6, and 5.7). Female neurogenesis patterns were similar between breeding conditions, except for %DCX+ cover, which was higher in breeding condition than in post-breeding condition, a seasonal difference in an unexpected direction.

5.4.1 Volumes

HVC and RA volume results were consistent with female blackbirds singing less than males with singing being most frequent in breeding condition (Nero 1956, Beletsky 1983, Kirn et al. 1989) and female cowbirds not singing at all (King and West 1990, Hamilton et al. 1997). Projections from HVC to RA are part of the caudal motor pathway of the SCS required for song production and I found species differences in females for both of these nuclei. First, breeding female blackbirds a higher proportion of brains with a discernable HVC than breeding female cowbirds and female blackbirds showed an increase in the proportion of brains with a discernable HVC from post-breeding to breeding condition (Table 5.1). Second, for RA volume, the effect of breeding condition

was greater in blackbirds, suggesting that both male and female blackbirds underwent an increase in RA volume whereas this effect of breeding condition was dampened in cowbirds because only male cowbirds underwent an increase in RA volume (Figure 5.4B). Finally, for RA volume, there was a greater sex difference in cowbirds than in blackbirds, which I expected because both sexes of blackbirds sing whereas only male cowbirds sing (Table 5.2). Together, these volumetric differences between species suggest that female blackbirds produce more song in breeding condition than in post-breeding condition and sing more than female cowbirds, which is consistent with previous studies on song rates (Nero 1956, Beletsky 1983, Kirn et al. 1989, King and West 1990).

Males had larger HVC and RA than females (Table 5.2, Figures 5.4A, 5.4B), which was previously reported in blackbirds (Kirn et al. 1989) and in cowbirds (Hamilton et al. 1997). Male blackbirds and cowbirds sing more than females and multiple studies have shown a positive correlation between sex differences in singing rate and sex differences in song control nuclei (Ball et al. 1994, Brenowitz 1997), even after taking phylogenetic relationships into account (MacDougall-Shackleton and Ball 1999). In addition, the female/male HVC size ratio increases as the female/male singing ratio increases from species in which females do not sing at all (i.e., cowbirds) to species in which females sing but males sing more (i.e., blackbirds) to duetting species (i.e., wrens) (Hall et al. 2010).

The relative size of HVC and RA decreased in post-breeding condition (Table 5.2, Figures 5.4A, 5.4B) and that was especially true in males, which has been reported in blackbirds (Kirn et al. 1989), but has not been previously investigated in cowbirds.

Nottebohm (1981) was the first to show this dramatic seasonal increase in HVC and RA, which are due to changes in cell sizes, cell spacing and cell numbers (Tramontin and Brenowitz 2000). I found that, in males, HVC increased by 196% in cowbirds and 243% in blackbirds whereas RA increased by 143% in cowbirds and 224% in blackbirds (Figure 5.4). The increase in HVC volume in blackbirds is similar to the 288% increase in HVC size in spotted towhees (*Pipilo maculatus*), which is among the greatest volumetric increase reported in a song control nucleus (Smith 1996). Indeed, this seasonal brain plasticity in songbirds is likely the most pronounced of any adult vertebrate and the volume of the song control system and singing behaviour have been shown to be seasonally plastic in every seasonally breeding songbird studies so far (reviewed by Tramontin and Brenowitz 2000). The mechanism that stimulates this seasonal plasticity is increasing day length that begins at the end of winter, which stimulates gonadal recrudescence and increases circulating levels of gonadal sex steroids, particularly testosterone (Smith et al 1997, Bernard and Ball 1997). Indeed, testosterone mediates seasonal changes in the song control nuclei (Tramontin and Brenowitz 2000). HVC and RA contain steroid receptors (Arnold et al. 1976, Brenowitz and Arnold 1992, Smith et al. 1996) and castration greatly decreases seasonal increases in these nuclei (Gulledge and Deviche 1997, Smith et al. 1997), whereas exogenous testosterone re-establishes these volumetric increases in castrated males (Nottebohm 1980, Bernard and Ball 1997). Finally, seasonal patterns of circulating testosterone mirrors the seasonal growth of song control nuclei (Smith 1996, Smith et al. 1997, Brenowitz et al. 1998), which I showed in blackbirds and cowbirds (Tables 5.2, Figure 5.4, Chapter 4).

Telencephalon volume decreased in post-breeding condition (Table 5.2, Figure 5.4C). A meta-analysis by Smulders (2002) and more recently a study by De Groof et al. (2009) using magnetic resonance imaging showed similar results. Functionally, decreasing brain matter in post-breeding condition could reduce the costs of maintaining energetically expensive brain tissue at a time when food is scarce (Yaskin 1984, Smulders 2002). It is unlikely that cell numbers change seasonally in the telencephalon, therefore changes in telencephalon volume could be caused by changes in neuropil, cell bodies and/or extracellular space, all mechanisms that are not mutually exclusive (Smulders 2002, De Groof et al., 2009). However, no study that I am aware of has found support for possible proximate and ultimate causes for these reported decreases in telencephalon volume in post-breeding condition.

5.4.2 Neurogenesis

DCX has only recently been used extensively to measure neurogenesis in the adult avian brains, however it is accepted as a reliable endogenous marker of neurogenesis (Balthazart and Ball 2014a, b). Very few studies have simultaneously examined sex and seasonal changes in neurogenesis in general, or with DCX in particular. In the current study, I measured the density of DCX+ cells and fibres in fields of view within the HVC. However, without stereology looking at the entire HVC, I cannot conclude that the total number of cells in the HVC differs between groups relative to outside the HVC.

My results on sex differences in DCX+ are opposite from those previously reported in canaries in which males had more DCX+ migrating cells than females

(Balthazart et al. 2008, Balthazart and Ball 2014b). Female blackbirds and cowbirds had higher levels of neurogenesis than males, based on all three DCX+ density measurements (%DCX+ cover, number of round cells, and number of fusiform cells; Table 5.3, Figures 5.5, 5.6, 5.7), a female-biased sex difference that is also present in starlings (Hall and MacDougall-Shackleton 2012). A negative correlation between singing rate and neurogenesis was also reported in male canaries that sing less in the presence of females, but show more neurogenesis in HVC (Balthazart et al. 2008, Alward et al. 2014). The telencephalon, which includes HVC, broadly and highly expresses DCX, therefore the default neurogenesis levels in the telencephalon of blackbirds and cowbirds may be high (Boseret et al. 2007). More neurogenesis in HVC is thus associated with less singing, which is consistent with the lower rate of singing in female blackbirds relative to males (Nero 1956, Beletsky 1983, Kirn et al. 1989) and no singing in female cowbirds (King and West 1990). Male canaries had higher DCX immunoreactivity inside the HVC than just outside ventral to the HVC (Balthazart and Ball 2014 a,b), whereas %DCX+ cover was generally greater outside the HVC than inside in male blackbirds and cowbirds (Figures 5.2, 5.5). Thus, patterns of sex differences in neurogenesis and neurogenesis levels inside the HVC relative to outside the HVC seem to be species-specific.

Neurogenesis levels in male blackbirds and cowbirds were highest in post-breeding condition (Table 5.3, Figures 5.5, 5.6, 5.7). My results are consistent with previous studies showing that ongoing neurogenesis in HVC causes seasonal increases in neuron number, along with volume, and neurogenesis is at its peak in the autumn (Tramontin and Brenowitz 2000). Elevated testosterone and estrogen levels reduce the turnover rate of neurons in HVC and increase the survival of new neurons, thus

increasing the number of total neurons in HVC in the breeding season (Rasika et al. 1994, Hidalgo et al. 1995, Tramontin and Brenowitz 1999). Neurogenesis in HVC generates new RA-projecting neurons and interneurons, replacing old cells (Paton et al. 1985, Kirn and Nottebohm 1993). Peak neuron turnover in the autumn coincides with a peak in song learning and a reduction in song stereotypy in the canary, an open-ended song learner (Kirn et al. 1994). However, this seasonal peak in neuron turnover and a drop in song stereotypy is also present in an age-limited learner that does not change its song in adulthood (Tramontin and Brenowitz 1999), suggesting that peak neuron turnover may be more closely associated with song stereotypy only or that neuron turnover may be necessary for song learning, but not sufficient on its own (Tramontin and Brenowitz 2000). Both cowbirds (King and West 1988) and blackbirds (Marler et al. 1972, Yasukawa et al. 1980) are open-ended learners, so the post-breeding season may be a time during which song modification is at its maximum. Male cowbirds had more migrating cells in their HVC than male blackbirds (Table 5.3, Figure 5.7). This species difference in neuronal recruitment may be explained by cowbird males continuously modifying their song in response to stimulation by females (King and West 1988, Hamilton et al. 1997), whereas female input has not been reported to influence male blackbird song. In contrast, blackbirds had more migrating cells in post-breeding condition than in breeding condition, whereas no seasonal effect was present in cowbirds (Table 5.3, Figure 5.7). In sum, although neurogenesis peaked in post-breeding males of both species, it may be more concentrated in post-breeding condition in blackbirds than in cowbirds.

In contrast to males, the density of differentiating and fusiform cells in females remained similar between breeding conditions, but %DCX+ cover was greater in breeding condition (Table 5.3, Figures 5.5, 5.6, 5.7). This seasonal effect in %DCX+ cover was mainly driven by female blackbirds (Figure 5.5). Female blackbirds sing two song types; one is for pair-bond maintenance and the other is apparently territorial (Beletsky 1983). Unlike males that use their song to attract mates and thus would require peak song performance established before the breeding season commences, female blackbirds may need to modify their pair-bonding song during the breeding season based on the mate they choose for that year. Song modification in breeding female blackbirds may be the reason why female blackbirds have more migrating cells per field of view than female cowbirds (Table 5.3, Figure 5.7).

5.4.3 Summary and conclusions

Female blackbirds and cowbirds sing less than males and had smaller HVC and RA with higher levels of neurogenesis in HVC relative to the adjacent nidopallium. In male cowbirds and blackbirds, the volumes of HVC and RA increased in breeding condition when androgen levels were high and neurogenesis in HVC was greatest in post-breeding condition, when androgen levels were low. Larger volumes of HVC and RA in breeding males may allow them to sing frequently to attract mates, whereas higher levels of neurogenesis in post-breeding males allow them to modify and prepare their songs for the upcoming breeding season. Although the volumes of HVC and RA did not change seasonally in females, breeding female blackbirds had more discernable HVC with more HVC neurogenesis than non-breeding female blackbirds, suggesting that the function of song in female blackbirds may be different than in males. Female blackbirds may need to

show the most song plasticity in breeding condition, especially to accommodate pair-bonding. Female cowbirds do not sing at all and thus fewer cowbird females had a discernable HVC than female blackbirds. In conclusion, song control nuclei volume and neurogenesis change with sex and season in two icterid species and reflect differences in song rate, song function, and song plasticity, closely connecting behaviour with the brain.

5.5 References

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Chapter 6

6. General Discussion⁶

6.1 Summary

In this thesis, I found that cognition and the brain of a brood parasite were closely associated with its behavioural ecology, results that are consistent with the underlying hypothesis in neuroecology (Table 6.1).

In Chapters 2 and 3, I tested brown-headed cowbirds (*Molothrus ater*) on two types of spatial tasks in non-breeding and breeding conditions. In Chapter 2, I used a large-scale foraging task that required subjects to move through their environment and remember a specific location for 24 h, like cowbirds would in the wild. I found that females made fewer errors and took more direct paths to the rewarded location than males. There was no effect of breeding condition on either females or males. In Chapter 3, I used operant conditioning to test both spatial and colour memory with touchscreens, which required birds to remember a location or a colour for just a few seconds. The touchscreen task differed from the foraging task in spatial scale, retention interval, and in the response required of the birds (i.e., approaching a cup versus pecking a shape on a screen). The touchscreen task was meant to test whether female cowbirds have overall better spatial memory than males or if sex differences in spatial memory in cowbirds is

⁶ This chapter has not been published.

Table 6.1. Summary of sex (F = females, M = males) differences in cognition and the brain of brown-headed cowbirds (BHCO) and red-winged blackbirds (RWBL). For brevity, only one measure of neurogenesis was included: % doublecortin immunoreactive (DCX+) cover, with a higher percentage indicating higher neurogenesis.

Cognitive or neural variable	Sex difference?	Species difference?	Chapter #
Spatial memory: Food cups ¹	BHCO: F > M	n/a	2
Spatial memory: Touchscreens ²	BHCO: M > F	n/a	3
Colour memory: Touchscreens ²	BHCO: F = M ³	n/a	3
Hippocampus : Volume	BHCO and RWBL: F > M	BHCO > RWBL	4
Hippocampus : %DCX+ cover	BHCO: F > M RWBL: F = M	BHCO > RWBL	4
HVC : Volume	BHCO and RWBL: M > F	BHCO = RWBL	5
RA : Volume	BHCO: M > F ⁴ RWBL: M > F	BHCO = RWBL	5
HVC : %DCX+ cover	BHCO and RWBL: F > M	BHCO = RWBL	5

¹ Task required subjects to move through space and locate a previously-baited food cup.

² Task required subjects to remember a location or a colour in their immediate visual field.

³ Both females and males performed better on the spatial task than on the colour task.

⁴ The effect of sex is greater in cowbirds than in blackbirds.

task-dependant. On the spatial touchscreen task, males performed better than females in two ways. First, breeding males outperformed breeding females. Second, at the shortest retention interval, males outperformed females. There was no sex difference on the non-spatial (colour) task, however, a seasonal effect was observed in females only, with performance increasing from non-breeding to breeding conditions. Both females and males performed better on the spatial touchscreen task than on the colour touchscreen task.

In Chapter 4, I studied the neural basis of spatial memory, the hippocampus. I studied the effects of sex, season, and species on hippocampal volume and neurogenesis. Unlike Chapters 2 and 3, I was able to include a nonparasitic relative, the red-winged blackbird (*Agelaius phoeniceus*) in my analyses. I found that hippocampal size, relative to the telencephalon, was greater in cowbirds than in blackbirds and greater in females than in males. Similarly, doublecortin immunoreactivity (DCX+), an endogenous marker of neurogenesis, was higher in the hippocampus of cowbirds than that of blackbirds, relative to the telencephalon. However, unlike the volume analyses, there was a significant sex by species interaction in %DCX+ cover, with neurogenesis being greater in female cowbirds than in male cowbirds, but no sex difference in blackbirds. Breeding condition did not affect the size of the hippocampus, but it did affect neurogenesis, which was highest in non-breeding cowbirds with no seasonal effects in blackbirds.

In Chapters 2-4, I examined the cognitive and neural aspects of spatial memory, whereas Chapter 5 focused on the neural basis of bird song. I measured the size of the HVC and the robust nucleus of the arcopallium (RA) and neurogenesis in HVC to

determine whether these variables vary with signing rate in breeding and post-breeding female and male cowbirds and blackbirds. HVC and RA were larger in males than in females and in males and HVC and RA were larger in breeding condition than in non-breeding condition. High levels of neurogenesis in HVC were associated with lower singing rates; females, who sing less than males, had more HVC neurogenesis and non-breeding males, who sing less than breeding males, also had more HVC neurogenesis. Breeding female blackbirds, who sing, had more discernable HVC than female cowbirds, who do not sing at all.

There are many novel features presented in this thesis. First, this is one of few studies examining spatial memory in a brood parasite and the only study to examine both sex and seasonal changes in spatial memory in a brood parasite. This is only the second study to examine cognition in a parasite using operant conditioning with touchscreens. Third, this is one of few studies to simultaneously examine sex and seasonal changes in the size and neurogenesis of the hippocampus and song control nuclei. Fourth, this is one of few studies to use doublecortin as a marker of neurogenesis and the only study to use doublecortin in a brood parasite or an icterid. In all, my thesis has made a multi-faceted contribution to the field of neuroecology.

6.2 Seasonal stability in spatial memory and the hippocampus

Spatial memory and hippocampus size and neurogenesis were enhanced in females and in cowbirds and, except for neurogenesis, were seasonally stable. Female

cowbirds performed better than males on the food cup task in Chapter 2, regardless of breeding condition. Similarly, in Chapter 4, hippocampus size remained stable across breeding conditions, with cowbirds and females having a larger hippocampus, relative to the telencephalon, than blackbirds and males, respectively. Species differences in hippocampal neurogenesis were similar to those of hippocampal volume, with cowbirds having more hippocampal neurogenesis than blackbirds. However, only female cowbirds had more hippocampal neurogenesis than male cowbirds as opposed to females of both species having a larger hippocampus than males. Spatial memory and hippocampus volume in brown-headed cowbirds were not plastic as I had initially predicted. Although this thesis alone cannot conclude that cowbirds, especially females, have adaptively specialized hippocampi and spatial memory for their brood-parasitic mode of reproduction, the results are consistent with this hypothesis. I only included cowbirds in Chapter 2 whereas, in Chapter 4, I included cowbirds and a closely-related non-parasite, the red-winged blackbird, in the analyses. As Garland and Adolph (1994) noted, however, at least three species should be included in studies that aim to determine whether a trait is an adaptation. Studies like this thesis, along with future studies addressing similar questions, could together provide a clear picture of adaptations of the brain and cognition in brood parasites.

6.3 Resemblance to food-storing songbirds

Patterns of spatial cognition and hippocampus volumes in cowbirds resemble patterns reported in food-storing birds (Table 6.1). A seasonally stable hippocampus volume has also been reported in food-storers (Hoshooley and Sherry 2004, Hoshooley et

al. 2007). Smulders et al. (1995) reported a larger hippocampus in chickadees in the autumn, although no other study has been able to replicate these findings. In addition to a seasonally stable hippocampus, food-storers have a larger hippocampus than non-food-storers (Krebs et al. 1989, Sherry et al. 1989, Hampton et al. 1995, Garamszegi and Eens 2004, Lucas et al. 2004) and perform better on various types of spatial memory tasks (Balda and Kamil 1989, Kamil et al. 1994, Olson 1991, Olson et al. 1995, McGregor and Healy 1999). Non-food-storers also generally perform equally well on spatial and non-spatial (i.e., colour) tasks, whereas food-storers perform better on spatial memory tasks (Brodbeck and Shettleworth 1995). Although I did not include a non-parasitic relative in my cognitive tests, cowbirds performed better on the spatial touchscreen task than on the colour touchscreen task (Chapter 3), suggesting that their spatial memory, like in food-storers, may be enhanced relative to other forms of memory (Brodbeck and Shettleworth 1995). In support, cowbirds also had a larger hippocampus than blackbirds (Chapter 4). Selection on one sex (i.e., females) can generate species differences. Indeed, because female and male cowbirds share most of their genes, a trait favored in one sex can occur in both (Lande 1980, Wyman et al. 2013). However, female cowbirds performed better on the foraging task, which more closely resembled host nest searching in the field. Thus, although spatial memory in general may be enhanced in both male and female cowbirds relative to other forms of memory, which is associated with a larger relative hippocampus (Chapters 3 and 4), the specialized form of spatial memory that may be required to remember the location of host nests appears to be enhanced in females only (Chapter 2).

6. 4 Sex differences are dependent on task type

Unlike food-storers that outperform non-food-storers on a variety of tasks, sex differences in cowbird spatial cognition are dependent on task type (Table 6.1). Females performed better than males on the food cup task (Chapter 2), whereas males performed better than females on the touchscreen task (Chapter 3). Selection for enhanced spatial memory for cache sites acts on both sexes in a food-storing species, whereas selection for enhanced spatial memory of host nests likely only acts on females. Thus, the effect of food-storing on spatial memory and the hippocampus is expected to be stronger than that of brood parasitism.

Like female cowbirds, male mammals perform better than females on spatial tasks requiring movement through space (Hampson 2002). Male humans, meadow voles, and laboratory rats perform better than females on spatial memory tasks in which the subject or the external stimuli are in motion (Gaulin and Fitzgerald 1986, Williams et al. 1990, Silverman et al., 2000, Postma et al., 2004). From an ultimate perspective, this male advantage in spatial navigation is thought to stem from males ranging more widely than females (Gaulin and Fitzgerald 1986, Hampson 2002, 2008). The Fertility and Parental Care Theory proposes that females must maintain a threshold body fat to maintain ovulatory competence and to preserve the implanted embryo, which both promote reproductive success (Sherry and Hampson 1997, Hampson 2002). To maintain this required body fat, females must reduce long distance ranging that is more typical of males (Hampson 2002). In addition, mammals are usually polygynous and thus males navigate longer distances than females (Hampson 2002, Gaulin and Fitzgerald 1986). In cowbirds, it is not the mating system that influences space use, but the need for females

to remember the location of suitable host nests within their egg-laying range (Rothstein et al. 1986). In sum, space use and spatial memory in female cowbirds resembles those of male mammals.

Like male cowbirds, female humans outperform males on stationary spatial memory tasks (Hampson 2002). Females consistently perform better than males when there is no dynamic change in the objects or the subject and dynamic positions of the objects do not need to be visualized. This sex difference is normally shown when the subject is presented with an array of objects in their immediate visual field and is asked which objects changed positions after a retention interval. Multiple studies have reported such a female-bias in object location memory (Eals and Silverman 1994, James and Kimura 1997, McBurney et al. 1997, Voyer et al. 2007). Why might female humans be better at object location memory? Females, who may not range long distances to maximize reproductive fitness (see above), may be adapted to foraging near their home and navigate in a familiar setting where landmarks are reliable spatial cues (James and Kimura 1997, Hampson 2002). Indeed, females rely more on landmarks for spatial navigation in humans and rodents (Galea and Kimura 1993, Sandstrom et al. 1998, Williams et al. 1990; reviewed in Hampson 2002). Thus, male cowbirds outperform female cowbirds on a task in which female humans normally excel at relative to males.

In cowbirds, I reported a reversal of typical sex differences in spatial memory generally reported in mammals. Female cowbirds were better at the spatial memory task requiring movement through space (Chapter 2), like male mammals, whereas male cowbirds were better at a stationary object location memory task (Chapter 3), like female mammals. Space use in female cowbirds (host nest visits) is analogous to space use seen

in male mammals (visiting multiple mates, foraging longer distances) and thus, likely explains the female-biased sex difference I reported in Chapter 2. However, the basis for the male-biased sex difference in Chapter 3 is more difficult to understand. From an ultimate perspective, there may be a trade-off associated with specialization in a particular form of memory because enhanced cognitive function has metabolic and life history costs (Hasenstaub et al., 2010, Burns et al., 2011, Cole et al. 2012). Enhanced performance by female cowbirds on large-scale navigational tasks may come at a cost to stationary spatial task. Alternatively, there could be functional incompatibility between these two types of spatial memory (Sherry and Schacter 1987). From a proximate perspective, improved male spatial performance on the touchscreen task could be due to increased androgen concentrations. Androgen concentrations in males increased a great deal more between non-breeding and breeding conditions than in females and it was only in breeding condition that males outperformed females (Chapter 2). Elevated androgens has been shown to increase spatial memory performance in mammals and songbirds, which may have also caused male cowbirds to increase their spatial performance from non-breeding to breeding condition and outperform breeding female cowbirds (more details below; Galea et al. 1996, Hodgson et al. 2008). In conclusion, I have demonstrated a reversal of sex-typical spatial memory performance in a species with sex-role-reversed use of space.

6.5 Seasonal changes in stationary spatial memory and hippocampal neurogenesis

Although breeding condition did not affect spatial memory of female and male cowbirds on the food cup task (Chapter 2) and females on the touchscreen task (Chapter 3), it did affect performance of males on the touchscreen task. Males had more accurate spatial memory when they were in breeding condition and when their circulating androgen levels (mainly testosterone) were at their highest (Chapter 3). Enhanced spatial memory or acquisition of a spatial task has been associated with high testosterone levels in both mammals and birds (Galea et al. 1996, Hodgson et al. 2008). Breeding male deer mice (*Peromyscus maniculatus*) showed better acquisition of a spatial task than females, when their testosterone levels are highest, whereas no sex difference existed in non-breeding condition (Galea et al. 1996). In a spatial touchscreen task similar to my study, great tits improved their performance after they were administered exogenous testosterone (Hodgson et al. 2008). The hippocampus of songbirds expresses high levels of the enzyme aromatase, which converts testosterone into estradiol (Saldanha et al. 2004). Aromatase produces high levels of local estradiol in the hippocampus, which binds to estrogen receptors and enhances spatial memory acquisition and performance (Oberlander et al. 2004, Hodgson et al. 2008, Bailey et al. 2013). Thus, enhanced spatial performance by breeding male cowbirds is probably caused by elevated testosterone, which is converted to estradiol in the hippocampus.

Although breeding condition did not affect the volume of the hippocampus, it did affect hippocampal neurogenesis, especially in cowbirds (Chapter 4). Neurogenesis

peaked in non-breeding condition, when cowbirds are not searching for host nests and memory load is low. Barnea and Nottebohm (1994) also reported a peak hippocampal neurogenesis in black-capped chickadees in the autumn, however this peak coincided with a time of the year when spatial memory load was presumed to be at a peak. There have been inconsistencies with seasonal differences in hippocampal neurogenesis in food-storers as Hoshooley and Sherry (2004) found no seasonal variation in the production of new neurons. Hippocampal neurogenesis in food-storing birds could vary between years and more specifically relate to factors such as food availability, energy balance, and flock dominance structure (Sherry and MacDougall-Shackleton 2014). Seasonal changes in hippocampal neurogenesis in cowbirds from the current study resemble patterns exhibited by the polygynous Richardson's ground squirrel (*Urocitellus richardsonii*) (Burger et al. 2014). Although home ranges are larger in the breeding season, especially for males that mate with multiple females within their home range, hippocampal neurogenesis is at a peak in non-breeding condition (Burger et al. 2014). Hippocampal neurogenesis in the cowbirds also resembles a consistent pattern of seasonal neurogenesis observed in male songbirds. Neurogenesis into the song control nuclei HVC is at a peak in the non-breeding season (reviewed by Tramontin and Brenowitz 2000). Singing rate peaks in the breeding season, therefore neurogenesis in non-breeding may allow birds to practice and prepare their song for the upcoming breeding season (Tramontin and Brenowitz 2000). Similarly, heightened hippocampal neurogenesis in non-breeding cowbirds may allow them to refresh their memory and prepare their brains for new spatial information in the upcoming breeding season.

6.6 Song control nuclei

Although the relative hippocampus size was larger in female blackbirds and cowbirds (Chapter 4), the relative size of the song control nuclei, HVC and RA, were larger in male blackbirds and cowbirds (Chapter 5). This male-biased sex difference is correlated with higher singing rates in males than in females (reviewed by Hall et al. 2010). Various studies, across many species have shown a positive correlation between sex differences in song control nuclei sizes and sex differences in singing rate (Ball et al. 1994, Brenowitz 1997), even after taking phylogenetic relationships into account (MacDougall-Shackleton and Ball 1999). Female cowbirds do not sing at all (King and West 1990, Hamilton et al. 1997), whereas female blackbirds sing, but less than males (Nero 1956, Beletsky 1983, Kirn et al. 1989). Consistent with this species difference in females, 1) fewer female cowbirds had an HVC that was discernable from the surrounding telencephalon than female blackbirds, especially in the breeding season and 2) there was a greater sex difference in RA volume in cowbirds than in blackbirds (Chapter 5).

Female blackbirds and cowbirds had higher relative neurogenesis in HVC than males, which is consistent with previous work on starlings (Hall and MacDougall-Shackleton 2012) but opposite from previous work in canaries (Balthazart et al. 2008). However, in male canaries, a lower singing rate was associated with higher HVC neurogenesis (Alward et al. 2014). So, it follows that female blackbirds and cowbirds, that sing less than males, would have higher HVC neurogenesis. However, hippocampal neurogenesis was also higher in females than males, but females, especially female cowbirds, have higher a spatial memory load than males (Chapter 4). Thus, sex

differences in neurogenesis and how they relate to the use of the brain region in question are not fully understood.

There was strong seasonal plasticity in the volumes of HVC and RA (Chapter 5). HVC and RA were approximately twice as large in the breeding season relative to the non-breeding season (Chapter 5). Breeding condition had a greater effect on RA volume in blackbirds than in cowbirds, which points to two non-mutually-exclusive mechanisms. First, the effect of breeding condition is dampened in cowbirds because only male cowbirds, the only sex that sings, undergo a volumetric increase in RA. Second, female choice is a strong feature of sexual selection of song in cowbirds (King and West 1988, Hamilton et al. 1997) whereas male-male competition is the stronger feature of sexual selection in blackbirds (Marler et al. 1972, Yasukawa et al. 1980). Male-male competition of territories in blackbirds occurs solely in the breeding season whereas male cowbirds can modify their song in response to stimulation by females at any time of the year.

Like the cowbird hippocampus (Chapter 4), I found that neurogenesis in the HVC was highest in non-breeding condition (Chapter 5). So, in both HVC and the hippocampus, cowbirds produce new neurons at a time of the year when these brain regions are used the least. Although seasonal patterns of hippocampal neurogenesis are not consistent across studies, increases in HVC neurogenesis are quite consistently in the non-breeding season, when birds are practicing and modifying their songs (reviewed by Tramontin and Brenowitz 2000).

6.7 Neurogenesis: forgetting or acquisition of new memories?

The HVC of male blackbirds and cowbirds and the hippocampus of cowbirds, especially females, are presumably less relied upon in post-breeding condition. However, neurogenesis was at a peak at this time of year in the HVC of male blackbirds and cowbirds and in the hippocampus of cowbirds. These results suggest that seasonal fluctuations of neurogenesis in an individual regulates forgetting. If the role of neurogenesis was primarily to allow acquisition of new memories, neurogenesis would have peaked in the breeding season. Neurogenesis replaces old cells which may erase memories at a time of the year when past memories are no longer required (Barnea and Pravosudov 2011, Tramontin and Brenowitz 1999). Purging past memories will presumably reduce interference when new memories must be acquired in the breeding season (Hoshooley and Sherry 2007, Barnea and Pravosudov 2011). Indeed, in mice, reducing neurogenesis increased memory persistence (Akers et al. 204). Forgetting indirectly allows male blackbirds and cowbirds, which are open-ended learners, to produce a newly perfected song and female cowbirds to remember the location of new host nests independently from last year's experiences.

6.8 Previous cognitive and neural work on cowbirds

A previous thesis, Lattanzio (2007), examined sex differences in spatial and colour memory of brown-headed cowbirds on touchscreens like in Chapter 3, but did not test cowbirds on a spatial navigational task like Chapter 2. Also, unlike my thesis,

Lattanzio (2007) did not vary breeding condition. Although Lattanzio (2007) did not find a sex difference on the colour task, like with my thesis, she found that females outperformed males on the spatial touchscreen task. This discrepancy is difficult to explain, especially considering that my thesis and hers found significant effects of sex in opposite directions. One difference between my thesis and Lattanzio (2007) may be motivation. The birds in Lattanzio (2007) were not tested 7 days a week, frequently ceased to respond during testing, and performed only slightly above chance. In addition, Lattanzio (2007) did not food deprive her birds at the same level as I did, which may have played an important role in causing differences in motivation between her study and mine. Finally, Lattanzio (2007) used fewer birds and, due to stochastic events, may have led to a Type I error. Nonetheless, as my thesis has demonstrated, our understanding of sex differences in avian cognition remains imperfect and there may be other factors that we do not yet understand that account for differences between the two studies.

Sherry et al. (1993) previously reported that breeding female cowbirds had a larger hippocampus than breeding males whereas no sex difference existed in blackbirds. However, Lattanzio (2007) did not report a sex difference in the hippocampus volume of cowbirds. Although I recorded a significant female-biased effect of sex in cowbirds, this effect of sex was also present in blackbirds. In the South American congeneric shiny cowbird, females have a larger hippocampus than males (Reboreda et al. 1996), however, these results have not been replicated as Clayton et al. (1997) used the same individuals in their seasonal study. Thus, patterns of sex differences in the hippocampus size of brood-parasitic and non-brood-parasitic species are not as consistent as patterns in food-storing and non-food-storing birds.

I did not record an effect of breeding condition on hippocampus size, even with a large sample size relative to previous studies on cowbirds (Lattanzio 2007, Clayton et al. 1997). However, Lattanzio (2007) found that female brown-headed cowbirds had a smaller hippocampus later in the summer, when breeding was nearly done, than at the peak of breeding in the spring, whereas no seasonal effect was reported in males. Similarly, the congeneric shiny cowbird has a larger hippocampus in the breeding season than in the non-breeding season (Clayton et al. 1997). Thus, seasonal differences in the hippocampus size of cowbirds are not consistent across studies.

Similar to my study, female cowbirds in Lattanzio (2007) had more hippocampal neurogenesis than males, however, unlike my study, hippocampal neurogenesis was greatest in breeding condition. I recorded high hippocampal neurogenesis in non-breeding condition (Chapter 4), just like the song control nuclei HVC (Chapter 5). No other study has investigated seasonal and sex effects of hippocampal neurogenesis in cowbirds and thus further work is needed.

The volumes of the HVC and RA have previously been shown to be larger in males than females (Hamilton et al. 1997, Lattanzio 2007) and larger in males in the breeding season (Lattanzio 2007). Thus, sex and seasonal patterns of song control nuclei sizes are consistent across studies, including mine. Sex and seasonal changes in HVC neurogenesis had not been previously examined in cowbirds, but are consistent with data on other songbirds (Tramontin and Brenowitz 2000).

6.9 Future work

My thesis has made a solid contribution to the field of neuroecology. However, many other unanswered questions remain. To test whether brood parasitism has specifically caused an evolutionary enlargement of the hippocampus and increased hippocampal neurogenesis, we need to conduct large-scale phylogenetic analyses like those that exist for food-storing behaviour (e.g., Garamszegi and Eens 2004, Lucas et al. 2004). Thus, we should include other brood parasites such as Eurasian cuckoos (family Cuculidae), honeyguides (Piciformes), and whydahs (Viduidae) in a large-scale study. In addition, hippocampal measurements should be taken across breeding conditions. To increase power, it would be very useful to develop magnetic resonance imaging (MRI) techniques to allow for repeated measures across breeding conditions and eliminate the necessity of euthanizing individuals. MRI would also allow researchers to investigate cognition in conjunction with brain work.

Data on detailed space use in the wild is lacking in cowbirds. Cowbirds are too small to carry global positioning system (GPS) loggers that record fine-scale habitat use with high temporal resolution. With time, smaller and better technology may become available. For now, a radio-receiver array could be used to track the movement of female and male cowbirds in their egg-laying ranges. We could determine precisely how frequently female cowbirds visit host nests before, during and after parasitism. The use of MRI described above would be highly valuable, as we could take repeated measure of the hippocampus volume throughout the breeding season to see if hippocampus volume is correlated with spatial memory load of a given individual or even between individuals.

Females that make more visits to more host nests and that navigate in more spatially complex terrain may have larger hippocampi.

In my thesis, I did not count the number of neurons in the hippocampus, which could change seasonally within individuals or by sex or species between individuals. Indeed, changes in cell number may help explain more subtle changes in behaviour that volume cannot detect (Roth et al. 2010). To better understand the neural mechanisms of memory and evolution, volume, in conjunction with cell numbers, should be incorporated into hippocampal analyses (Roth et al. 2010).

6.10 Conclusions

I used an integrative approach to determine the effects of sex and breeding condition on cognition and the brain of the brood-parasitic brown-headed cowbird and a nonparasitic relative, the red-winged blackbird. Studies outlined in my thesis contribute many new findings to the fields of cognition, neuroscience, and physiology. In cowbirds, I showed (i) a female-biased sex difference on a navigational memory task resembling host nest visits, (ii) a female-biased sex difference in hippocampus size and neurogenesis, and (iii) that both cognitive and relative hippocampus size measures were stable between breeding conditions. On a touchscreen spatial memory task, breeding males had both the highest performance and the highest androgen levels of all groups, suggesting that hormones may regulate cognitive abilities. Female and male cowbirds performed better on this spatial task than on a similar colour task, suggesting that cowbirds may have enhanced spatial memory relative to other forms of spatial memory. Consistent with this finding, cowbirds had a larger hippocampus with more neurogenesis than blackbirds.

Finally, sex and seasonal differences in the size and neurogenesis of song control nuclei were positively and negatively associated with singing rate, respectively. In sum, sex and seasonal differences in cognition and the brain were closely associated with spatial and singing behaviours observed in the wild.

6.11 References

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- Sherry, D. F., Vaccarino, A. L., Buckenham, K., & Herz, R. S. (1989). The hippocampal complex of food-storing birds. *Brain, Behavior and Evolution*, 34, 308-317.
- Sherry, D. F., Forbes, M. R., Khurgel, M., & Ivy, G. O. (1993). Females have a larger hippocampus than males in the brood-parasitic brown-headed cowbird. *Proceedings of the National Academy of Sciences of the United States of America*, 90, 7839-7843.
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- Smulders, T. V., Sasson, A. D., & DeVogd, T. J. (1995). Seasonal variation in hippocampal volume in a food-storing bird, the black-capped chickadee. *Journal of Neurobiology*, 27, 15-25.
- Tramontin, A. D., & Brenowitz, E. A. (2000). Seasonal plasticity in the adult brain. *Trends in Neurosciences*, 23, 251-258.
- Voyer, D., Postma, A., Brake, B., & Imperato-McGinley, J. (2007). Gender differences in object location memory: A meta-analysis. *Psychonomic Bulletin & Review*, 14, 23-38.
- Williams, C. L., Barnett, A. M., & Meck, W. H. (1990). Organizational effects of early gonadal secretions on sexual differentiation in spatial memory. *Behavioral Neuroscience*, 104, 84-97.

- Wyman, M. J., Stinchcombe, J. R., & Rowe, L. (2013). A multivariate view of the evolution of sexual dimorphism. *Journal of Evolutionary Biology*, 26, 2070-2080.
- Yasukawa, K., Blank, J. L., & Patterson, C. B. (1980). Song repertoires and sexual selection in the red-winged blackbird. *Behavioral Ecology and Sociobiology*, 7, 233-238.

Appendix 1: Animal Use Protocol Approval for Chapters 2-5

2007-001-03::7:

AUP Number: 2007-001-03

AUP Title: Neurogenesis, Spatial Memory, and Animal Cognition

Yearly Renewal Date: 08/01/2014

The YEARLY RENEWAL to Animal Use Protocol (AUP) 2007-001-03 has been approved, and will be approved for one year following the above review date.

1. This AUP number must be indicated when ordering animals for this project.
2. Animals for other projects may not be ordered under this AUP number.
3. Purchases of animals other than through this system must be cleared through the ACVS office.
Health certificates will be required.

REQUIREMENTS/COMMENTS

Please ensure that individual(s) performing procedures on live animals, as described in this protocol, are familiar with the contents of this document.

The holder of this Animal Use Protocol is responsible to ensure that all associated safety components (biosafety, radiation safety, general laboratory safety) comply with institutional safety standards and have received all necessary approvals. Please consult directly with your institutional safety officers.

Submitted by: Kinchlea, Will D
on behalf of the Animal Use Subcommittee

Curriculum Vitae

Mélanie F. Guigueno, BSc (Honours), MSc, PhD Candidate

Department of Biology and Advanced Facility for Avian Research
University of Western Ontario, London, Ontario, Canada

Research fields

Behavioural ecology, cognition, neuroscience

Academic training

- 2011–present **PhD Candidate**, Department of Biology, University of Western Ontario
Supervisors: Dr. David Sherry and Dr. Scott MacDougall-Shackleton
Thesis title: Sex and seasonal differences in cognition and the brain in an avian brood parasite, the brown-headed cowbird (*Molothrus ater*)
Anticipated defense date: April 2015
- 2007–2010 **Master of Science**, Department of Zoology, University of Manitoba
Supervisor: Dr. Spencer G. Sealy
Thesis title: Acceptance or rejection of cowbird parasitism: cues used in decision-making by yellow warblers (*Dendroica petechia*)
- 2003–2007 **Bachelor of Science (Honours)**, Department of Zoology
University of Manitoba and Université de Saint-Boniface
Supervisor: Dr. Spencer G. Sealy
Thesis title: Role of nest sanitation in the egg burial behaviour of yellow warblers (*Dendroica petechia*)

Publications (* work completed with a co-supervised undergraduate student)

18. Guigueno MF, MacDougall-Shackleton SA, Sherry DF. Accepted. Sex differences in spatial memory in brown-headed cowbirds: males outperform females on a touchscreen task. *PLoS One*.
17. Watt D, Sealy SG, Guigueno MF. In press. Antoine Joseph Lottinger's first book on the European cuckoo and its fosterers: a rare and little-cited book with three title-pages. *Archives of Natural History*.
16. Miller A, Elliott JE, Elliott KH, Guigueno MF, Wilson LK, Lee S, Idrissi A. 2015 Brominated flame retardant trends in aquatic birds from the Salish Sea region of the west coast of North America, including a mini-review of recent trends in marine and estuarine birds. *Science of the Total Environment* 502: 60-69.
15. Miller A, Elliott JE, Elliott KH, Guigueno MF, Wilson LK, Lee S, Idrissi A. 2014. Spatial and temporal trends in brominated flame retardants in seabirds from the Pacific coast of Canada. *Environmental Pollution* 195:48-55.
14. Guigueno MF, Sealy SG, Westphal* AM. 2014. Rejection of parasitic eggs in passerine hosts: Size matters more for a non-ejecter. *Auk: Ornithological Advances* 131:583-594.

Publications (continued)

13. **Guigueno MF**, Snow* DA, MacDougall-Shackleton SA, Sherry DF. 2014. Female cowbirds have more accurate spatial memory than males. *Biology Letters* 10: 20140026.
 12. **Guigueno MF**, Elliott KH, Levac J, Wayland M, Elliott JE. 2012. Differential exposure of alpine ospreys to mercury: Melting glaciers, hydrology or deposition patterns? *Environment International* 40: 24–32.
 11. **Guigueno MF**, Sealy SG. 2012. Increased investigation of manipulated clutches suggests egg recognition without rejection in a brown-headed cowbird (*Molothrus ater*) host, the yellow warbler (*Setophaga petechia*). *Auk* 129: 1–9.
 10. **Guigueno MF**, Sealy SG. 2012. Nest sanitation in passerine birds: implications for egg rejection in hosts of brood parasites. *Journal of Ornithology* 53: 35–52. (review)
 9. Capper* C-L, **Guigueno MF**, Sealy SG. 2012. Acceptance of simulated cowbird parasitism in a northern population of red-winged blackbirds. *American Midland Naturalist* 167: 127–135.
 8. Elliott JE, Levac J, **Guigueno MF**, Shaw DP et al. 2012. Factors influencing legacy pollutant accumulation in osprey: Biology, topography or melting glaciers? *Environmental Science and Technology* 46: 9681–9689.
 7. Jacobs SR, Elliott KH, **Guigueno MF**, Gaston AJ et al. 2012. Determining seabird body condition using non-lethal measures. *Physiological and Biochemical Zoology* 85: 85–95.
 6. Rasmussen JL, Sealy SG, **Guigueno MF**, Elliott KH. 2012. Infrequent ejection of artificial bronzed cowbird (*Molothrus aeneus*) eggs by the clay-colored thrush (*Turdus grayi*) in Costa Rica. *Ornitologia Neotropical* 23: 33–41.
 5. **Guigueno MF**, Sealy SG. 2011. Aggression towards egg-removing cowbird elicits clutch abandonment in parasitized yellow warblers, *Dendroica petechia*. *Animal Behaviour* 81: 211–218.
 4. Sealy SG, **Guigueno MF**. 2011. Cuckoo chicks evicting their nest mates: coincidental observations by Edward Jenner in England and Antoine Joseph Lottinger in France. *Archives of Natural History* 38: 220–228.
 3. Stewart RLM, **Guigueno MF**, Sealy SG. 2011. How small is too small? Incubation of large eggs by a small host. *Canadian Journal of Zoology* 89: 968–975.
 2. **Guigueno MF**, Sealy SG. 2010. Clutch abandonment in parasitized yellow warblers: Egg burial or nest desertion? *Condor* 112: 399–406.
 1. **Guigueno MF**, Sealy SG. 2009. Nest sanitation plays a role in egg burial by yellow warblers. *Ethology* 115: 247–256.
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Media Interests

BBC Earth: “Devious ways birds fight egg wars,” April 10, 2015

Phys.org: “Female brown-headed cowbirds perform spatial tasks better than males,” February 28, 2014

The Globe and Mail (National newspaper): “Bird study finds females have a better sense of direction,” February 26, 2014

Découverte, Radio-Canada (Canadian Broadcasting Corporation): “Mémoire d’oiseau,” January 27, 2013

Portland Press Herald, Maine, USA: “Birding: Fascinating findings gathered here in Maine,” July 11, 2010

Academic Honours and Awards

J. D. Detweiler Award, University of Western Ontario, August 2014, \$1,500

Ontario Graduate Scholarship with Distinction (“distinction” awarded to top 20 university-wide candidates), University of Western Ontario, May 2013–April 2014, \$16,500

Natural Sciences and Engineering Research Council of Canada (NSERC) Doctoral Postgraduate Scholarship, University of Western Ontario, January–December 2011 and 2012, \$21,000 per annum (\$42,000 total)

E. Scherer Memorial Scholarship, University of Manitoba, 2009–2010, \$10,000

G. A. Lubinsky Memorial Scholarship, University of Manitoba, 2008–2009, \$4,350

Roger Evans Memorial Scholarship, University of Manitoba, 2008–2009, \$3,000

NSERC Master’s Canada Graduate Scholarship, University of Manitoba, 2007–2008, 2008–2009, \$17,500 per annum (\$35,000 total)

Delta Marsh Scholarship, University of Manitoba, May 2008, \$500

H. E. Welch Award, University of Manitoba, June 2007, \$650

David Ian MacKenzie Medal, University of Manitoba, June 2007, Medal and \$150

NSERC Undergraduate Summer Research Award, University of Manitoba, May–August 2007 and May–August 2006, \$5,625 per summer

American Ornithologists’ Union Student Membership Award, University of Manitoba, January–December 2006, no monetary value

Isbister Scholarship, Université de Saint-Boniface, 2004–2005, \$750

University 1/Dean’s Honour List, Université de Saint-Boniface and University of Manitoba, 2003–2007, no monetary value

Bursary of Excellence, Université de Saint-Boniface, 2003–2004, 2004–2005, \$1,000 per annum (\$2,000 total)

Official Minority Language Award, Université de Saint-Boniface, 2003–2004, 2004–2005, \$500 per annum (\$1,000 total)

Research Grants

Sigma Xi Grants-in-Aid of Research, University of Western Ontario, May–December 2013, \$665

Animal Behavior Society Student Research Grant, University of Western Ontario, May– December 2013, \$2,000

Graduate Thesis Research Award, University of Western Ontario, January–August 2013, \$1065

American Ornithologists' Union Research Award, University of Western Ontario, May– December 2012, \$2,500

F. M. Memorial Chapman Research Grant, Western Michigan University, May–December 2010, \$800

Society of Canadian Ornithologists Taverner Student Research Award, University of Manitoba, May–December 2009, \$1,000

Research Presentations (presenter and first author, asterisks indicate poster)

International Ornithological Congress 2014, Tokyo, Japan, August 2014

Animal Behaviour Society Meeting 2014, Princeton, USA, August 2014

Behaviour 2013, NewcastleGateshead, United Kingdom, August 2013

The Fifth North American Ornithological Conference*, Vancouver, Canada, August 2012

Behavior 2011, Bloomington, Indiana, July 2011

American Ornithologists' Union/Cooper Ornithological Society/Society of Canadian Ornithologists Joint Meeting, San Diego, USA, February 2010

The 31st International Ethological Conference*, Rennes, France, August 2009

Biological Sciences Graduate Students' Association Seminar Series, University of Manitoba, Winnipeg, Manitoba, February 2009

American Ornithologists' Union/Cooper Ornithological Society/Society of Canadian Ornithologists Joint Meeting, Portland, USA, August 2008

Prairie Universities Biological Symposium, Winnipeg, Manitoba, February 2008

Biological Sciences Graduate Students' Association Seminar Series, University of Manitoba, Winnipeg, Manitoba, October 2007

NSERC Student Poster Presentation*, Winnipeg, Manitoba, September 2007

Prairie Universities Biological Symposium, Regina, Saskatchewan, February 2007

Association francophone pour le savoir, Université de Saint-Boniface, Winnipeg, Manitoba, April 2005

Invited presentations

Bird Café, Rikkyo University, Tokyo, Japan, August 2014

Ruthven is for the Birds Festival, Ruthven Park National Historic Site, September 2013

Association francophone pour le savoir, Université de Saint-Boniface, Winnipeg, Manitoba, February 2008

Research Experience (in addition to Honours, MSc, and PhD work)

Field worker, Canada-Brazil Research Program with Drs. Cristovam Diniz and David Sherry

- Banded giant cowbirds and recorded their behaviour at host nests (caciques)
- Universidad Federal do Para, Brazil
- January–February 2013

Contractor, Environment Canada, contract number K8A30-10-0144

- Data analysis and manuscript write up for a multi-year project on contaminants in ospreys
- September–December 2010

Field and laboratory worker, Hormones and Behaviour Project with Dr. Sharon Gill

- Examined the effects of hormones on egg rejection in a cowbird host
- Western Michigan University, USA
- May–August 2010

Contractor, Environment Canada, contract number K8A30-09-0201

- Sample preparation for stable isotope analysis
- January–March 2010

Natural History Researcher and Translator, collaboration with Dr. Spencer Sealy

- Translated and researched 18th century books (French to English) on brood parasitism
- Traveled to France to conduct research in libraries and museums
- November 2008–August 2009

Field worker, Tropical studies project with Dr. Spencer Sealy

- Analyzed the acceptance and rejection responses of hosts of bronzed cowbirds
 - Cerro de la Muerte, Costa Rica
 - April 2008
-

Teaching Experience (in French and English)

Teaching Assistant, Biology 2290G Scientific Methods in Biology, University of Western Ontario, London, Ontario, January–April 2015, January–April 2014, May–August 2013, January–April 2013

Teaching Assistant, Biology 1001A Biology for Sciences I, University of Western Ontario, London, Ontario, September–December 2014, May–August 2013

Guest Lecturer, Universidade Federal do Pará, Bragança, Brazil, February 2013

Laboratory coordinator and teaching assistant (2 sections), BIOL 1021 Biologie I, Université de Saint-Boniface, Winnipeg, Manitoba, September–December 2010

Laboratory coordinator and teaching assistant, BIOL 2231 L'évolution et la structure morphologique des chordés, Université de Saint-Boniface, Winnipeg, Manitoba, January–April 2010

Guest Lecturer, Introductory Ecology, Department of Biological Sciences, University of Manitoba, Winnipeg, Manitoba, March 2009

Laboratory coordinator and teaching assistant, ZOOL 4240 Biology of Birds, University of Manitoba, Winnipeg, Manitoba, September–December 2008

Teaching Assistant (2 sections), ZOOL 2600 Introductory Invertebrate Zoology, University of Manitoba, Winnipeg, Manitoba, September–December 2007

Leadership

Resident Assistant for 15 undergraduate researchers (volunteer), Pierce Cedar Creek Institute, Hastings, Michigan, May–August 2010

Supervised over 30 undergraduate field and laboratory assistants and co-supervised the following six Honours students:

Arthur, Justin. In progress. Sex and seasonal differences in numerical ability of brown-headed cowbirds, *Molothrus ater*. Department of Biology, University of Western Ontario.

Coto, Alex. 2014. Brown-headed cowbirds can count: Numerical ability of brood parasite. Department of Biology, University of Western Ontario.

de la Chevotière, Jennifer. 2014. Tracking GnRH plasticity through the seasons in two blackbird species. Department of Psychology, University of Western Ontario.

Torok, Zsombor. 2013. Reward type influences the sexes differently on a spatial memory task in brown-headed cowbirds. Department of Psychology, University of Western Ontario.

Snow, Danielle. 2012. Sex differences in spatial working memory of brown-headed cowbirds. Department of Psychology, University of Western Ontario.

Capper, Christie-Leigh. 2010. Acceptance of simulated cowbird parasitism in a northern population of red-winged blackbirds. Department of Biological Sciences, University of Manitoba.

Service

Journals Refereed

Auk: Ornithological Advances
Animal Behaviour
Behavioral Ecology and Sociobiology
Behaviour
Canadian Journal of Zoology
Ecology, Ethology & Evolution
Ethology
Journal of Avian Biology
PLoS ONE
Western Birds
Wilson Journal of Ornithology

Organizing committee member, Ontario Ecology, Ethology, and Evolution
Colloquium, University of Western Ontario, London, Ontario, May 2013

Bird bander (volunteer), University Field Station (Delta Marsh), Delta Marsh,
Manitoba, August–September 2008, July–October 2009

Organizing committee member, Biological Sciences Graduate Students' Seminar
Series, Department of Biological Sciences, University of Manitoba, Winnipeg,
Manitoba, September 2008– April 2009

Christmas Bird Count (volunteer), December 2007 and 2008 (Manitoba), December
2009 (Guatemala), December 2014 (Ontario and British Columbia)

Organizing committee member, Prairie Universities Biological Symposium, University
of Manitoba, March 2007–February 2008
