EFFECT OF A TERRITORIAL CHALLENGE ON THE STEROID PROFILE
OF A JUVENILE SONGBIRD

by

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ABSTRACT

Adult male song sparrows (*Melospiza melodia*) display territorial aggression year-round. Territoriality is supported by gonadal sex steroids during the breeding season but might be supported by brain-derived sex steroids during the non-breeding season. Juvenile (hatch-year) male song sparrows also defend territories during the non-breeding season despite having immature testes and low plasma testosterone levels. Juvenile males therefore represent an excellent model to investigate the modulatory role of neurosteroids on aggression. Here, free-living non-breeding juvenile males experienced a 10-min simulated territorial intrusion (STI) or control condition. Blood and brain were collected, and steroids were quantified in blood and microdissected brain regions via liquid chromatography-tandem mass spectrometry (LC-MS/MS). Juveniles were robustly aggressive during an STI. Juveniles were equally as aggressive or more aggressive than non-breeding adults. Androgens and estrogens were low or non-detectable in blood and brain regardless of social context. After an STI, progesterone and glucocorticoids were elevated in the blood and brain in a region-specific manner. Together, these data suggest that juvenile non-breeding aggression might be rapidly modulated by adrenal-derived or brain-derived progestogens and glucocorticoids. These rapid changes in steroid levels might impact behaviourally relevant neural circuits and mobilize energy stores from peripheral organs. To our knowledge, we are the first to investigate the interplay between developmental life-stage and social context at the behavioural and steroidal level in an avian model. This work provides insight into distinct endocrine and neuroendocrine mechanisms of social behaviour in a juvenile songbird that might extend to other vertebrates.
Lay Summary

Aggression and the physiological mechanisms that support aggression are highly evolutionarily conserved. Like mammals, birds rely on steroid hormones like testosterone to engage in aggressive social interactions. However, few studies have examined how steroids and their effects on aggression might shift across development. In the current thesis, I investigate how a short aggressive interaction rapidly affects steroids in the blood and brain of juvenile male birds. First, I found that juvenile birds are aggressive, like adult males. Second, I found that juveniles exposed to an aggressive interaction do not have elevated levels of testosterone or estrogens and might not rely on them to support aggressive behaviour. Instead, my data suggest that juveniles might rely on steroids produced in the adrenal glands or brain to support aggression. This work provides new insights into the potential role of adrenal-derived and brain-derived steroids in modulating social behaviour in juvenile birds.
PREFACE

This thesis is the original work of the author, Sofia Gray. I carried out all field and laboratory experiments at the University of British Columbia (Vancouver Campus) under the supervision of Dr. Kiran Soma. All methods and protocols used in completion of this thesis were approved by the Animal Care Committee at the University of British Columbia (Certificate number A16-0234).

I was personally responsible for data collection, formal data analyses, figure drafting, and manuscript writing for the behavioural and steroid data presented in completion of this thesis. Cecilia Jalabert trained me on field and laboratory techniques necessary to perform the pertinent experiments. Jalabert also aided in rating subjects’ aggression for the behavioural study. Tissues were collected with the help of Jalabert, Athena Varghese, and Emma Lam. Lam also aided in sample processing. Dr. Chunqi Ma conducted steroid quantification by liquid chromatography-tandem mass spectrometry. Dr. Soma was the supervisory author on this project and provided guidance on conceptualization, methodology, and manuscript writing.

An earlier version of Chapters 2 and 3 were presented at the following two conferences: UBC Multidisciplinary Undergraduate Research Conference (2022) and the Society for Behavioral Neuroendocrinology Annual Meeting (2022). Asterisks (*) indicate presenting author.

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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AH</td>
<td>Anterior hypothalamus</td>
</tr>
<tr>
<td>BnST</td>
<td>Bed nucleus of the stria terminalis</td>
</tr>
<tr>
<td>Cb</td>
<td>Cerebellum</td>
</tr>
<tr>
<td>CBG</td>
<td>Corticosteroid-binding globulin</td>
</tr>
<tr>
<td>CG</td>
<td>Central grey</td>
</tr>
<tr>
<td>DHC</td>
<td>11-Dehydrocorticosterone</td>
</tr>
<tr>
<td>DHEA</td>
<td>Dehydroepiandrosterone</td>
</tr>
<tr>
<td>E₂</td>
<td>17β-estradiol</td>
</tr>
<tr>
<td>HPG axis</td>
<td>Hypothalamic-pituitary-gonadal axis</td>
</tr>
<tr>
<td>LC-MS/MS</td>
<td>Liquid chromatography-tandem mass spectrometry</td>
</tr>
<tr>
<td>LS</td>
<td>Lateral septum</td>
</tr>
<tr>
<td>NAc</td>
<td>Nucleus accumbens</td>
</tr>
<tr>
<td>NCM</td>
<td>Caudomedial nidopallium</td>
</tr>
<tr>
<td>POA</td>
<td>Preoptic Area</td>
</tr>
<tr>
<td>RIA</td>
<td>Radioimmunoassay</td>
</tr>
<tr>
<td>SDMN</td>
<td>Social decision-making network</td>
</tr>
<tr>
<td>StAR</td>
<td>Steroidogenic acute regulatory protein</td>
</tr>
<tr>
<td>STI</td>
<td>Simulated territorial intrusion</td>
</tr>
<tr>
<td>TnA</td>
<td>Nucleus taeniae of the amygdala</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<td>--------------</td>
<td>--------------------------------------------------</td>
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<tr>
<td>VMH</td>
<td>Ventromedial hypothalamus</td>
</tr>
<tr>
<td>VTA</td>
<td>Ventral tegmental area</td>
</tr>
<tr>
<td>5α-DHT</td>
<td>5α-dihydrotestosterone</td>
</tr>
<tr>
<td>3β-HSD</td>
<td>3β-hydroxysteroid dehydrogenase</td>
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This thesis would not be possible without the continued support of my supervisor, Dr. Kiran Soma. I’d like to sincerely thank him for his incredible mentorship and for sharing his expertise with me during my time at the lab. Kiran’s dedication, enthusiasm, and advice pushed me to develop my research and critical-thinking skills, ultimately helping me become the scientist I am today. For his continued support, I am extremely grateful.

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1. INTRODUCTION

1.1 STEROID MODULATION OF AGGRESSION

Aggression is a highly conserved social behaviour observed across taxa as it is extremely important for survival and reproduction. Agonistic encounters are observed when two or more individuals fight for access to limited resources necessary for survival, such as food. Aggression encompasses physical confrontations among individuals and displays of threatening behaviour, which are often employed to avoid physical conflict (Soma et al., 2008). Furthermore, most intrasexual aggression among conspecifics is typically observed during periods of breeding when individuals fight to attain or maintain territories and mates (Göppert et al., 2016; Heimovics et al., 2015b; Knapp & Moore, 1995; Soma et al., 2008).

Steroid hormones are, in part, responsible for supporting aggressive behaviour (Soma, 2006; Soma et al., 2008). The endocrine modulation of aggression has been examined in nonhuman primates (Honess & Marin, 2006; Sapolsky, 1982), rodents (Heimovics et al., 2015b; Munley et al., 2018; Trainor & Marler, 2001), teleost fish (Göppert et al., 2016; Jalabert et al., 2015; Pradhan et al., 2014; Quintana et al., 2016; Silva & Pandolfi, 2019), reptiles (Knapp & Moore, 1995) and birds (Bergeon Burns et al., 2013; Fokidis et al., 2013; Levin & Wingfield, 1992; Lischinsky & Lin, 2020; Rosvall, 2013; Soma, 2006). Sex steroids, such as testosterone, are implicated in supporting territorial aggression. Sex steroids are regulated by the hypothalamic-pituitary-gonadal (HPG) axis. In brief, the hypothalamus releases gonadotropin-releasing hormone. This peptide hormone then acts on the anterior pituitary gland to signal the release of luteinizing hormone and follicle-stimulating hormone, which travel through the bloodstream to the gonads. Ultimately, androgens and estrogens are synthesized in the gonads.
and released into the bloodstream to produce powerful effects on organs and tissues throughout the body, including the brain (Balthazart et al., 2018). The ‘challenge hypothesis,’ first proposed by John Wingfield in 1990, describes the intimate relationship between sex steroids and male-male aggression. According to the ‘challenge hypothesis,’ baseline levels of blood testosterone are elevated during the breeding season compared to the non-breeding season (Wingfield et al., 1990). Moreover, testosterone increases beyond baseline breeding levels only during periods of social instability, such as during territory establishment. During periods of social stability, however, blood testosterone levels remain at breeding baseline (Hirschenhauser & Oliveira, 2006; Wingfield et al., 1990).

Despite support for the role of circulating androgens and estrogens in modulating territorial aggression in breeding contexts, some species maintain aggression year-round. In these species, aggression seems to be independent of circulating androgens and estrogens and therefore cannot be explained fully by the ‘challenge hypothesis.’ In many ways, significantly reducing the production of gonadal-derived steroids during the non-breeding season is advantageous.

Testosterone has a variety of deleterious fitness costs when maintained at high systemic levels for extended periods of time. These include an increase in energetic costs, stress, and suppression of the immune system (Soma et al., 2008; Wingfield et al., 2001). In dark-eyed juncos (*Junco hyemalis*), for example, testosterone-treated males suffer higher mortality rates over the winter, possibly because testosterone treatment causes elevated corticosterone and reduced fat stores (Ketterson et al., 1996). When blood testosterone levels are maintained below the maximal physiological concentrations, the negative health and survival impacts that are associated with chronic high androgens are curtailed.
Song sparrows (*Melospiza melodia*) are one model species that demonstrate year-round territoriality. The subspecies *Melospiza melodia morph* is a resident (non-migratory) population in British Columbia, Washington, Oregon, and California. Song sparrows are socially monogamous and have multiple broods of young with the same partner during the spring and summer months. As suggested by the ‘challenge hypothesis,’ blood testosterone levels in male song sparrows are highest during territory establishment and during periods of social instability among neighbors, usually occurring during the early breeding season. In the late breeding season, males are more focused on parental care and breeding baseline testosterone levels are lower. During this time, blood testosterone increases above baseline only during periods of social instability such as during a brief agonistic encounter with a new neighbor or during a predator interaction (Wingfield et al., 1990).

In male song sparrows, territoriality persists into the non-breeding season with only a brief lapse in aggression during molt when individuals dedicate energy to shedding old feathers and growing new ones. This represents a distinct shift away from the ‘challenge hypothesis’ because the testes regress during the non-breeding season and blood testosterone levels drop to non-detectable levels (Jalabert et al., 2021; Soma et al., 2008; Wingfield et al., 2019). The non-breeding winter months are difficult with low food abundance, low ambient temperatures, high precipitation, and short daylengths negatively impacting survival. There are high mortality rates among song sparrows, especially those that are unable to establish a territory (Arcese, 1989a; Arcese & Smith, 1988). Particularly high mortality rates are observed in juvenile (hatch-year) song sparrows who make their first attempt at establishing a territory during the non-breeding season.
While the fitness consequences of high systemic testosterone inform our understanding of why aggression and testosterone are decoupled during the non-breeding season, only recently has work shed light on the endocrine mechanisms that regulate non-breeding aggression. Steroids were classically thought to only bind to intracellular receptors, which change gene expression by binding to DNA. However, steroids are now known to also bind to membrane-bound receptors, where they produce potent and rapid non-genomic effects on physiology and behaviour by activating second messenger signaling cascades (reviewed in Wilkenfeld et al., 2018). Thus, steroids can act on much shorter timescales – minutes to hours instead of days to weeks (Heimovics et al., 2015b; Soma et al., 2008; Wilkenfeld et al., 2018). In the context of aggression, sex steroids like testosterone increase within minutes of engaging in an agonistic encounter with a conspecific territorial challenger (Heimovics et al., 2015a; Soma et al., 2008; Wingfield et al., 2019). With song sparrows in particular, castration does not decrease non-breeding aggression (Wingfield, 1994), but pharmacological inhibition of aromatase (CYP19A1), the enzyme responsible for converting androgens into estrogens, does reduce aggression in the non-breeding season (Soma et al., 1999; Soma et al., 2000b). Considered together, these lines of evidence suggest that testosterone and 17β-estradiol (E2), a major estrogen, support non-breeding aggression. However, these steroids are not found in circulation during the non-breeding season. The potent effects of testosterone and E2 on behaviour may therefore originate from within the brain.

1.2 NEUROSTEROIDS

In song sparrows, aromatase is highly expressed in the brain during both the breeding and non-breeding seasons (Balthazart et al., 2018; Soma et al., 2003, 2008). Furthermore, all
steroidogenic enzymes necessary for the conversion of cholesterol into estrogens are found in the brain of birds and other vertebrates (Jalabert et al., 2021; Schlinger & Remage-Healey, 2012) (Figure 1). Of particular interest is the social decision-making network (SDMN), a network of brain regions encompassing the social behaviour network and the mesolimbic reward system (O’Connell & Hofmann, 2011, 2012). The SDMN is intimately involved in the regulation of social behaviours such as mating, parental care, and aggression (Heimovics et al., 2012; Jalabert et al., 2021). This network has many dopaminergic projections, high sex steroid receptor expression, and high steroidogenic enzyme expression throughout (Goodson, 2005; Heimovics et al., 2016; Newman, 1999; O’Connell & Hofmann, 2011, 2012; Pradhan & Soma, 2012). Thus, it is very likely that the SDMN is capable of synthesizing steroids and is sensitive to steroid action. 3β-hydroxysteroid dehydrogenase (3β-HSD) is an enzyme that is present in the SDMN and is responsible for converting inactive androgens and progestogens into active androgens and progestogens respectively (Figure 1). In song sparrows, brain 3β-HSD is upregulated during the non-breeding season and rapidly increases following an acute aggressive interaction (Pradhan & Soma, 2012). The brain itself therefore contains all the enzymes necessary to rapidly synthesize steroids, termed neurosteroids, either de novo from cholesterol or from circulating prohormones that cross the blood brain barrier.
Figure 1. Pathway depicting steroidogenesis from cholesterol to the different classes of steroids, including progestogens, glucocorticoids, androgens, and estrogens. Steroids are bolded and enzymes responsible for the conversion of precursors to metabolites are listed next to arrows showing the direction of conversion. Figure courtesy of Melody Salehzadeh (2019).
In adult male song sparrows, seasonal shifts in neurosteroid levels have been investigated previously (Jalabert et al., 2021). At baseline, neural androgens and estrogens are found in distinct SDMN brain regions in the breeding season but not in the non-breeding season. Breeding levels of androstenedione and 5α-dihydrotestosterone (5α-DHT), a precursor and product of testosterone respectively, are two- to twenty-fold higher in some brain regions compared to in blood. Estrogens, while detected in some SDMN brain regions, are non-detectable in blood in breeding subjects. Thus, blood steroid levels are not necessarily reflective of local steroid levels in the brain. Furthermore, steroid levels can drastically vary between brain regions. Androgens and estrogens are not detected in the brain during the non-breeding season in adult male song sparrows that are not stimulated with an aggressive encounter (Jalabert et al., 2021). Recent work in non-breeding adult males found that subjects who experience a brief territorial challenge before capture show a rapid increase in brain, but not blood, testosterone (Jalabert et al., unpublished work). Furthermore, after experiencing a territorial challenge, both progesterone and corticosterone are elevated in blood and brain, with blood levels exceeding those in the brain (Jalabert et al., unpublished work). If neurosteroids are synthesized rapidly and only as needed during a non-breeding agonistic encounter, then they should only be detected during and immediately following an aggressive dispute.

Like adults, juvenile male song sparrows demonstrate high levels of non-breeding territorial aggression (Wingfield & Hahn, 1994a). Sparrows are considered juveniles, or hatch-year animals, during their first year post-hatching. They are identified by incomplete ossification of the skull and immature testes. Juveniles, born in summer, fledge and leave the nest in late summer and early fall. As molt subsides and the non-breeding season begins during late fall and early winter, juveniles attempt to establish their own territories. Although very little work has
investigated juvenile aggression, some reports have shown that juveniles are as aggressive as adults during their first non-breeding season (Arcese, 1989b; Soma et al., 2002; Soma & Wingfield, 2001). This may be because territory establishment is critical for survival, particularly for juveniles who experience extremely high mortality rates compared to adults (Arcese, 1989b; Arcese & Smith, 1988; Beecher, 2017; Nol & Smith, 1987; Sullivan, 1989; Templeton et al., 2012a). Establishing a territory provides access to food and shelter, which greatly impacts survival (Arcese & Smith, 1988; Nol & Smith, 1987). Juveniles that can establish a territory early in winter are much more likely to survive, reach sexual maturity, and reproduce in the following summer (Arcese, 1989b; Beecher, 2017; Beecher et al., 2000; Soma et al., 1999; Wingfield & Hahn, 1994b).

While there are limited observations showing similar behavioural patterns in juveniles and adults, even less is known about the steroid profile of juveniles in the context of aggression. Steroidogenic acute regulatory protein (StAR) and CYP11A1 are the rate-limiting protein and enzyme that convert cholesterol into pregnenolone and are expressed in the developing zebra finch brain and rat brain (London et al., 2006; Mellon, 2007; Figure 1). Likewise, CYP17A1, which converts progestogens into androgens, is expressed in the developing and adult zebra finch brain (London et al., 2003). CYP17A1 is also found in embryonic rodent brain (Mellon, 2007). Through CYP17A1, progesterone is converted into dehydroepiandrosterone (DHEA), an inactive androgen. DHEA is then converted into testosterone, an active androgen, via 3β-HSD which is expressed in the developing and adult zebra finch brain (London et al., 2006). In song sparrows, circulating DHEA levels measured by radioimmunoassay (RIA) are similar among adults and juveniles (Soma & Wingfield, 2001). However, circulating DHEA measured by liquid chromatography-tandem mass spectrometry (LC-MS/MS) has not been found at comparable
levels in adults or juveniles (Jalabert et al., 2021). LC-MS/MS is a much more specific, sensitive and accurate method for steroid measurement compared to antibody-based assays (Bertin et al., 2015; Jalabert et al., 2021; Penning et al., 2010). Thus, it is unclear if DHEA is present at meaningful levels that could affect behaviour in either adult or juvenile song sparrows. Because juveniles are sexually immature and their testes have likely not yet reached full functionality regarding steroid synthesis, it is unlikely that juvenile non-breeding aggression is supported by circulating androgens. Juveniles are therefore a good model to investigate the potential role of neurosteroids in modulating non-breeding aggression.

1.3 Measurement of Steroids by LC-MS/MS

Historically, steroids in circulation have been measured through antibody-based methods, such as RIA and enzyme-linked immunoassays. Although widely used, antibody-based assays suffer from various limitations. They often lack the specificity and sensitivity to quantify low steroid levels found in biological samples, particularly in the brain. Moreover, antibody-based assays can only measure one analyte per sample. These assays can also suffer from cross-reactivity between steroids that share similar chemical structures, which can lead to overestimates of steroid levels and disparities in the literature (Taylor et al., 2015).

LC-MS/MS, as mentioned, is the leading ultrasensitive technique in steroid measurement (Andrew & Homer, 2020; Bertin et al., 2015; Hamden et al., 2021; Koren et al., 2012; Taylor et al., 2015). With LC-MS/MS, multiple analytes of interest can be detected simultaneously in a single sample with extremely high specificity, sensitivity, and precision. Described in detail elsewhere (Hamden et al., 2021; Jalabert et al., 2021), the LC separates analytes of interest, such as steroids, based on polarity. Analytes are then ionized at an ionization source before the MS
system selects precursor ions based on their mass-to-charge ratio. These precursor ions are then fragmented into product ions, which are monitored by the MS. Product ions are again selected for based on their mass-to-charge ratio and then quantified by an ion detector. Because each analyte of interest has a unique retention time, mass-to-charge ratio, and fragmentation pattern of precursor to product ions, LC-MS/MS is much more specific than immunoassays. Because sex steroids, particularly those in the brain, can have profound physiological and behavioural effects even at extremely low concentrations, the high sensitivity of LC-MS/MS measurement is necessary to accurately capture the steroid environment and its powerful effects.

1.4 OBJECTIVES

Here, we aimed to characterize the behavioural and steroidal patterns of aggression in free-living juvenile male song sparrows during the non-breeding season. Using LC-MS/MS, we measured a panel of 12 steroids in blood, plasma, and microdissected brain regions (including nodes of the SDMN) in juveniles that were exposed to a simulated aggressive encounter or a control condition. Further, we compared male juvenile behaviour with non-breeding adult males. Together, these lines of evidence provide novel insights into the endocrine mechanisms of non-breeding aggression in a juvenile songbird.
2. MATERIALS AND METHODS

2.1 SUBJECTS

Free-living juvenile, or hatch-year, male song sparrows were captured in the non-breeding season (16 October 2019 to 20 November 2019; 19 November 2020 to 22 December 2020; 18 October 2021 to 27 October 2021). Song sparrows are considered juveniles, or hatch-years, during the non-breeding season after they have fledged from the nest. During this time, the skull is not yet fully ossified, and juveniles have not reached sexual maturity. The age of subjects was classified as juvenile if any part of the skull was not ossified (Figure 2). Subjects ranged in degree of ossification (Soma & Wingfield, 2001). All animal protocols and procedures followed guidelines set by the Canadian Council on Animal Care and were approved by the University of British Columbia Animal Care Committee.

FIGURE 2. OSSIFICATION OF THE SKULL

Figure 2. Representative skulls of varying degrees of ossification. Juveniles are born with unossified skulls (far left) that gradually ossify from the caudal regions to the rostral regions over the course of their first year post-hatching. Sparrows are considered adults when the skull is fully ossified (far right).
2.2 FIELD PROCEDURES & BEHAVIOUR MANIPULATION

Subjects were captured near Vancouver, British Columbia. Subjects’ territories were identified by visual and auditory tracking of individuals. Sometimes, a brief (<10 sec) song was played to aid in territory identification. After territory identification, a mist net (Avinet Research Supplies, Portland, ME, USA) was set up and furled in preparation for the behavioural study.

For the behavioural study, juvenile subjects were exposed to a simulated territorial intrusion (STI) (n = 15) or control (n = 11) condition and aggressive behaviours were monitored as previously described (Heimovics et al., 2016; Wingfield & Hahn, 1994a; Wingfield & Soma, 2002). STI subjects were exposed to a live caged decoy intruder with conspecific playback from a Bluetooth speaker for 10 min. During this time, response latency (time to first call, song, or flight from the focal subject), number of songs, number of flights, and time spent within 5 meters of the decoy were recorded. Additionally, call latency (time to first call from the focal subject), song latency (time to first song from the focal subject), and flight latency (time to first flight from the focal subject) were recorded. These behaviours have previously been identified as aggressive within this context (Heimovics et al., 2016; Wingfield & Hahn, 1994a; Wingfield & Soma, 2002). Control subjects were exposed to an empty cage and a silent speaker for 10 min and spontaneous displays of aggression were recorded.

Juvenile aggression was compared to that of non-breeding adult males exposed to an STI (n = 14) and a control condition (n = 15) who were captured for a separate study during the same non-breeding seasons (19 November 2020 to 22 December 2020; 18 October 2021 to 29 October 2021). Two observers rated behaviour for individual subjects (observer A rated behaviour between 16 October 2019 and 20 November 2019; observer B rated behaviour between 19 November 2020 and 22 December 2020 and 18 October 2021 to 29 October 2021). No
significant differences between observers were found for any behavioural markers of aggression (all p > 0.3).

After the STI or control period was complete, the mist net was unfurled and the caged decoy was removed from the focal subject’s territory. Subjects were then captured using a conspecific song playback that was played for a maximum of 5 min (STI: 51.47 ± 17.19 sec; control: 104.82 ± 25.20 sec; F(1, 24) = 3.501, p = 0.074). After capture, subjects were rapidly and deeply anaesthetized via isoflurane and quickly decapitated (< 3 min to avoid the effect of handling stress altering steroid levels; STI: 145.73 ± 5.49 sec; control: 149.18 ± 9.95 sec; F(1, 24) = 0.024, p = 0.878). Isoflurane exposure was minimized (approximately 20 to 30 sec) to avoid irritation and disruption to blood-brain barrier permeability (Tétrault et al., 2008). Further, isoflurane exposure was equal between groups. The brain was immediately dissected and snap-frozen on powdered dry-ice. Trunk blood was collected in heparinized microhematocrit tubes (Thermo Fisher Scientific, Waltham, MA, USA) and maintained on wet ice until returning to the laboratory. Trunk blood was then divided into two different aliquots: one aliquot was frozen as whole blood (termed blood hereafter). The other aliquot was centrifuged for 5 min into plasma, collected, and then frozen on dry ice. All samples were stored at -70°C until steroid extraction.

2.3 Brain microdissection

Flash-frozen brain was mounted to a cryostat mount using OCT compound (Thermo Fisher Scientific). Brains were sectioned at 300μm thick in the coronal plane on a cryostat at -13°C. A zebra finch brain atlas was used as a reference guide for major landmark identification, as previously described (Heimovics et al., 2016; Jalabert et al., 2021). Serial brain sections were
then mounted on cooled Superfrost Plus microscope slides (Thermo Fisher Scientific) and maintained at -70°C until microdissection.

The Palkovits punch technique was used to microdissect 11 brain regions including nodes within the SDMN: nucleus accumbens (NAc), preoptic area (POA), anterior hypothalamus (AH), lateral septum (LS), bed nucleus of the stria terminalis (BnST), ventromedial hypothalamus (VMH), nucleus taeniae of the amygdala (TnA), ventral tegmental area (VTA), and central grey (CG; Figure 3). Because it has high aromatase expression and is involved in song perception, we collected the caudomedial nidopallium (NCM). Because the cerebellum (Cb) has low aromatase expression, we collected it as a negative control. Brain sections containing the aforementioned regions were microdissected as previously described (Jalabert et al., 2021; Figure 3). Brain sections were microdissected over dry ice using a 1mm diameter stainless-steel biopsy punch tool (wet weight = 0.245 mg per punch; Integra Miltex, Princeton, NJ, USA). Each brain region was collected using a different punch tool and tools were thoroughly washed with deionized water and reagent alcohol between subjects to avoid cross-contamination of steroids between regions and subjects.
FIGURE 3: MICRODISSECATED BRAIN REGIONS

Figure 3. Representative locations of brain regions microdissected via Palkovits punch technique in the song sparrow brain (A-G). Coronal brain sections are presented from rostral to caudal. 1 mm punches are represented by circles. Figure adapted from Jalabert et al., 2021.

Abbreviations: AH, anterior hypothalamus; BnST, bed nucleus of the stria terminalis; Cb, cerebellum; CG, central grey; CoA, anterior commissure; LS, lateral septum; NAc, nucleus accumbens; NCM, caudomedial nidopallium; OM, tractus occipito-mesencephalicus; PC, posterior commissure; POA, preoptic area; TnA, nucleus taeniae of the amygdala; VMH, ventromedial hypothalamus; VTA, ventral tegmental area
Each brain region was collected using either four or six punches (0.98 or 1.47 mg respectively), except the NAc which was collected in one punch (1 punch, 0.245 mg; Figure 3). The NAc was punched along the midline in the section where Area X was most prominent. To collect the AH, two bilateral punches (4 punches) were taken from serial sections directly lateral to the midline and immediately ventral to the anterior commissure. The POA was collected in two serial sections along the midline in bilateral punches (4 punches) immediately anterior to the emergence of the anterior commissure. The LS sits between the lateral ventricles alongside the midline and was collected bilaterally in two serial sections (4 punches) where the anterior commissure emerges. Similarly, the BnST was collected as bilateral punches across two serial sections (4 punches) where the lateral ventricles terminate when the anterior commissure emerges. The VMH was collected bilaterally (4 punches) on the same two sections where the anterior commissure is present at the ventral end of the brain just lateral to the midline and ventral to the AH. The NCM was collected from three serial sections (6 punches) on and posterior to the section where the anterior commissure and the tractus occipito-mesencephalicus last appeared. Similarly, the TnA was also collected in three serial sections (6 punches) posterior to the disappearance of the anterior commissure and the tractus occipito-mesencephalicus. The VTA was collected bilaterally in two serial sections (4 punches) ventrolateral to the oculomotor nerve. The CG was bilaterally punched (4 punches) in serial sections immediately ventral to the posterior commissure and lateral to the midline. The Cb was collected across three serial sections in unilateral punches (6 punches total, 2 per section) from its first appearance onwards.

All punches were deposited into pre-chilled 2 mL polypropylene bead ruptor tubes (Sarstedt AG & Co., Nümbrecht, Germany) and maintained on dry ice during punching. Samples were stored at -70°C until steroid extraction and analysis.
2. 4 Steroid Extraction

We measured a panel of 12 steroids in blood (5 μL), plasma (10 μL), and microdissected brain tissue (0.245 to 1.47 mg depending on the number of punches). The panel included the following: progesterone, pregnenolone, corticosterone, 11-dehydrocorticosterone (DHC), DHEA, androstenedione, testosterone, 5α-DHT, E2, 17α-estradiol, estrone, and estriol. Plasma is often used to quantify circulating steroids because it is easier to process, but plasma overestimates circulating steroid concentrations (Taves et al., 2011). As such, blood was used to quantify steroids since blood more accurately represents circulating steroid levels. Plasma was processed alongside blood and brain for ease of comparison with previous reports of circulating steroids in song sparrows.

All samples were processed using liquid-liquid extraction as previously described (Hamden et al., 2021; Jalabert et al., 2021; Tobiansky et al., 2018, 2020). Five zirconium ceramic beads (1.4 mm in diameter; Thermo Fisher Scientific) were added to each bead ruptor tube. 50 μL of deuterated internal standard (progesterone-d9, pregnenolone-d4, corticosterone-d8, DHEA-d6, testosterone-d5, and E2-d4; C/D/N Isotopes Inc., Pointe-Claire, QC, Canada) were diluted in 50% high-performance liquid chromatography (HPLC)-grade methanol and added to each sample to track recovery and matrix interference. 1 mL of HPLC-grade acetonitrile (ACN) was added to each sample before samples were vortexed and homogenized at 4 m/s for 30 seconds using a bead mill homogenizer (Omni International Inc., Kennesaw GA, USA). Samples were then centrifuged at 16,100 g for 5 min. 1 mL of supernatant was extracted and transferred to borosilicate glass culture tubes that had been pre-cleaned with HPLC-grade methanol (VWR International, Radnor, PA, USA). 0.5 mL of HPLC-grade hexane was added to the extracted supernatant. Samples were briefly vortexed and centrifuged at 3,200 g for 2 min, then the hexane
was removed. The ACN phase was dried at 60°C for 45 min in a vacuum centrifuge (SPD111V; Thermo Fisher Scientific). After evaporation, samples were resuspended using 55 μL of 25% HPLC-grade methanol and transferred to 0.5 mL polypropylene microcentrifuge tubes (Thermo Fisher Scientific). Samples were centrifuged at 16,100 g for 2 min and 50 μL of supernatant were transferred to an LC vial insert (Agilent, Santa Clarita, CA, USA). Samples were stored at -20°C until injection into the LC-MS/MS.

Biological samples were extracted alongside a calibration curve of known amounts of steroid, blank controls, double blank controls, and duplicates of 2 pg and 200 pg quality controls. Accuracy and precision were assessed by measuring the coefficient of variation in quality control replicates. The calibration curve and quality controls were made from certified reference standards (Cerilliant Co., Round Rock, TC, USA) resuspended in 50% HPLC-grade methanol. Calibration curves ranged from 0.05 to 1000 pg per tube for progesterone, corticosterone, DHC, testosterone, 5α-DHT, estrone, 17α-estradiol, and estriol. The curve for androstenedione ranged from 0.1 to 1000 pg per tube while the E2 curve ranged from 0.2 to 1000 pg per tube. The curves for DHEA and pregnenolone ranged from 1 to 10000 pg per tube and 2 to 10000 pg per tube respectively.

2.5 Steroid Analysis by LC-MS/MS

Steroids were quantified using a QTRAP 6500 UHPLC-MS/MS system (Sciex LLC, Framingham, MA, USA) as previously described (Jalabert et al., 2021). Extracted samples were transferred to an autoinjector (15°C) and 45 μL per sample were injected into a Nexera X2 UHPLC system (Shimadzu Corp., Kyoto, Japan) and passed through an HPLC In-Line Filter (Phenomenex, Torrance, CA, USA) and a Poroshell 120 HPH C18 guard column (2.1 mm)
Samples were then separated on a Poroshell 120 HPH C18 column (2.1 x 50 mm; 2.7 μm; 40°C) using 0.1 mM ammonium fluoride in MilliQ water as mobile phase A (MPA) and HPLC-grade methanol as mobile phase B (MPB); the flow rate was 0.4 mL/min. MPB was at 10% for 0.5 min during sample loading. The MPA:MPB gradient profile then shifted to 42% MPB for 3.5 min (0.6 to 4 min). Next, the gradient was increased to 60% MPB until 9.4 min, which shifted to 60%–70% MPB between 9.4 to 9.5 min. Ultimately, it was ramped to 98% MPB until 11.9 min. A column wash was carried out at 98% MPB for 1.5 min (until 13.4 min). The MPB was then returned to starting conditions (10% MPB) for 1 min. The total run time was 14.9 min. The autoinjector needle was rinsed externally with 100% isopropyl alcohol before and after injection of each sample.

Each steroid was detected with two scheduled multiple reaction monitoring transitions; deuterated internal standards were detected with one multiple reaction monitoring transition. Progestogen, glucocorticoid, and androgen concentrations were acquired on a QTRAP 6500 UHPLC-MS/MS system (Sciex LLC) in positive electrospray ionization mode while estrogens were acquired in negative electrospray ionization mode. All water blanks were below the limit of detection. Intra-assay and inter-assay variation were determined by analyzing duplicates of 2pg and 200pg quality control samples within and between LC-MS/MS runs. The coefficient of variation for intra-assay precision was less than 20% for all steroids except DHC and androstenedione, whose intra-assay precision was less than 30%. The coefficient of variation for inter-assay precision was for each analyte of interest was less than 20%.

2.6 Statistical Analyses

Steroid concentrations were considered non-detectable when they fell below the limit of detection set by the calibration curves. For groups that had ≥ 40% detectable samples, missing
values were imputed based on left-censored quantile regression imputation using the METIMP web tool (Wei et al., 2018b; https://metabolomics.cc.hawaii.edu/software/MetImp/). This regression can accurately and robustly estimate values for biological samples that are missing not at random—i.e. samples that fall below the detection limit (Hamden et al., 2021; Jalabert et al., 2021; Wei et al., 2018a, b). For groups that had < 40% detectable samples, missing values were replaced with zero. To make comparisons between peripheral and brain steroid levels, 1 ml of blood was considered equivalent to 1.05 g, as before (Hamden et al., 2021). 14 data points were missing or excluded from analyses due to experimenter error or equipment malfunction.

Behaviour and steroid data were analyzed in R (version 4.1.2) with the following packages: tidyverse (version 1.3.1), rstatix (0.7.0), and psych (2.2.3). For both behaviour and steroid data, a Shapiro-Wilk test of normality was performed which revealed that data were not normally distributed (all p < 0.05). Therefore, data were either log-transformed prior to analyses or were analyzed with non-parametric tests. For behaviour, the effect of condition (STI or control) and age (juvenile or adult) on aggression were analyzed separately by non-parametric Mann-Whitney U tests.

Steroid data were analyzed in two ways. First, two-way mixed ANOVAs were performed on natural log-transformed data. Condition (STI or control) was coded as a between-subjects factor and region (blood and brain regions) was coded as a within-subjects factor to account for drawing multiple comparisons from the same subject. When appropriate, post-hoc pairwise comparisons with Benjamini-Hochberg multiple comparison correction were used to determine the source of significant main effects or interactions. Second, Spearman correlations with Benjamini-Hochberg correction were used to query the similarity between blood steroid concentrations and brain steroid concentrations among subjects exposed to an STI. The relation
between steroid level and aggressive behaviour were also examined using Spearman correlations with Benjamini-Hochberg correction. $P$ values $< 0.05$ were considered statistically significant. Figures were made in R (version 4.1.2) with the following packages: ggplot2 (3.3.5), PerformanceAnalytics (2.0.4), corrplot (0.92), and ggpubr (0.4.0). Figures were made using non-transformed data. Data are reported as mean ± SEM.
3. RESULTS

3.1 Behaviour

3.1.1 Effect of STI on Juvenile Aggression

There was a significant effect of STI on juvenile aggression (Figure 4). Mann-Whitney U tests revealed that juveniles exposed to an STI responded faster to playback compared to controls (\(W = 164, p < 0.0001\); Figure 4A). Call, song, and flight latency were also significantly affected by STI (Table 1). Juveniles exposed to an STI were quicker to call, sing, and fly compared to controls. Further, juvenile males exposed to an STI sang (\(W = 16.5, p < 0.001\)) and flew (\(W = 0, p < 0.0001\)) significantly more than control subjects (Figure 4B, 4C). STI juveniles also spent more time within 5 meters (\(W = 0, p < 0.0001\)) compared to controls (Figure 4D). This indicates that, overall, juveniles exposed to an STI were more aggressive than controls.
Figure 4. Aggressive displays performed by juvenile males during a 10-min simulated territorial intrusion (STI). STI juveniles (A) responded faster to playback, (B) sang, and (C) flew significantly more than controls. (D) STI juveniles also spent more time within 5 meters compared to controls. Juvenile Control n = 11, Juvenile STI n = 15. Data are mean ± SEM. *** p < 0.001.
**TABLE 1: EFFECT OF STI ON JUVENILE BEHAVIOURAL LATENCIES**

<table>
<thead>
<tr>
<th></th>
<th>STI</th>
<th></th>
<th>Control</th>
<th></th>
<th>W</th>
<th>p</th>
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<tr>
<td></td>
<td>Mean</td>
<td>SEM</td>
<td>Mean</td>
<td>SEM</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Call Latency (sec)</td>
<td>73.7</td>
<td>39.1</td>
<td>529.1</td>
<td>49.1</td>
<td>157.5</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>Song Latency (sec)</td>
<td>175.3</td>
<td>54.8</td>
<td>600.0</td>
<td>0.0</td>
<td>154.0</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>Flight Latency (sec)</td>
<td>55.7</td>
<td>12.4</td>
<td>572.7</td>
<td>27.3</td>
<td>165.0</td>
<td>&lt; 0.0001</td>
</tr>
</tbody>
</table>

**3.1.2 JUVENILE VERSUS ADULT AGGRESSION**

Juvenile male aggressive displays were similar to those of non-breeding adult males (Figure 5). A Mann-Whitney U test revealed that non-breeding STI juveniles and STI adults did not differ in the time to respond to playback ($W = 98.5, p = 0.7932$; Figure 5A). Call, song, and flight latency did not significantly differ between juveniles and adults (Table 2). Juveniles and adults who experienced an STI sang ($W = 107, p = 0.9478$) and flew ($W = 90, p = 0.5267$) at comparable levels (Figure 5B, 5C). However, juveniles exposed to an STI spent significantly more time within 5 meters compared to STI adults ($W = 57, p < 0.05$; Figure 5D). Non-breeding juveniles and adults exposed to a control condition did not differ in any behavioural parameters (data not shown, all $p > 0.05$).
Figure 5. Non-breeding juvenile and adult males were exposed to a simulated territorial intrusion (STI). (A) Response latency and the number of (B) songs and (C) flights did not differ by age. However, (D) STI juveniles spent significantly more time within 5 meters compared to adults. Juvenile STI n = 15, Adult STI n = 14. Data are mean ± SEM. * p < 0.05.
### TABLE 2: EFFECT OF AGE ON BEHAVIOURAL LATENCIES

<table>
<thead>
<tr>
<th></th>
<th>Juveniles</th>
<th></th>
<th>Adults</th>
<th></th>
<th></th>
<th>p</th>
</tr>
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<tr>
<td></td>
<td>Mean</td>
<td>SEM</td>
<td>Mean</td>
<td>SEM</td>
<td>W</td>
<td></td>
</tr>
<tr>
<td>Call Latency (sec)</td>
<td>73.7</td>
<td>39.1</td>
<td>78.8</td>
<td>41.0</td>
<td>117</td>
<td>0.6153</td>
</tr>
<tr>
<td>Song Latency (sec)</td>
<td>175.3</td>
<td>54.8</td>
<td>92.2</td>
<td>26.0</td>
<td>84.0</td>
<td>0.3709</td>
</tr>
<tr>
<td>Flight Latency (sec)</td>
<td>55.7</td>
<td>12.4</td>
<td>50.9</td>
<td>11.2</td>
<td>101.5</td>
<td>0.8957</td>
</tr>
</tbody>
</table>

### 3.2 STEROIDS IN BLOOD AND BRAIN

We measured a panel of 12 steroids in blood, plasma, and 11 brain regions, including regions in the SDMN, to examine the effect of STI on non-breeding juvenile males. Progesterone, corticosterone, DHC, and testosterone were detectable (≥ 40% of samples were detectable). All other steroids were considered non-detectable (< 40% of samples were detectable; Table 3).
# Table 3: Percentage of Detectable Samples by Group

<table>
<thead>
<tr>
<th></th>
<th>Progesterone</th>
<th>Corticosterone</th>
<th>DHC</th>
<th>Testosterone</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Blood</strong></td>
<td>Control: 91</td>
<td>100</td>
<td>100</td>
<td>27</td>
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<tr>
<td></td>
<td>STI: 93</td>
<td>100</td>
<td>93</td>
<td>36</td>
</tr>
<tr>
<td><strong>NAc</strong></td>
<td>Control: 0</td>
<td>91</td>
<td>0</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>STI: 7</td>
<td>100</td>
<td>0</td>
<td>20</td>
</tr>
<tr>
<td><strong>POA</strong></td>
<td>Control: 9</td>
<td>100</td>
<td>45</td>
<td>27</td>
</tr>
<tr>
<td></td>
<td>STI: 27</td>
<td>100</td>
<td>87</td>
<td>27</td>
</tr>
<tr>
<td><strong>AH</strong></td>
<td>Control: 0</td>
<td>100</td>
<td>36</td>
<td>18</td>
</tr>
<tr>
<td></td>
<td>STI: 33</td>
<td>100</td>
<td>60</td>
<td>20</td>
</tr>
<tr>
<td><strong>LS</strong></td>
<td>Control: 0</td>
<td>100</td>
<td>64</td>
<td>18</td>
</tr>
<tr>
<td></td>
<td>STI: 27</td>
<td>100</td>
<td>67</td>
<td>27</td>
</tr>
<tr>
<td><strong>BnST</strong></td>
<td>Control: 9</td>
<td>100</td>
<td>36</td>
<td>27</td>
</tr>
<tr>
<td></td>
<td>STI: 13</td>
<td>100</td>
<td>60</td>
<td>13</td>
</tr>
<tr>
<td><strong>VMH</strong></td>
<td>Control: 18</td>
<td>100</td>
<td>36</td>
<td>9</td>
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<tr>
<td></td>
<td>STI: 47</td>
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<td>33</td>
</tr>
<tr>
<td><strong>VTA</strong></td>
<td>Control: 0</td>
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<td>50</td>
</tr>
<tr>
<td></td>
<td>STI: 60</td>
<td>100</td>
<td>87</td>
<td>21</td>
</tr>
<tr>
<td><strong>CG</strong></td>
<td>Control: 9</td>
<td>91</td>
<td>82</td>
<td>27</td>
</tr>
<tr>
<td></td>
<td>STI: 60</td>
<td>100</td>
<td>93</td>
<td>21</td>
</tr>
<tr>
<td><strong>NCM</strong></td>
<td>Control: 9</td>
<td>100</td>
<td>100</td>
<td>36</td>
</tr>
<tr>
<td></td>
<td>STI: 27</td>
<td>100</td>
<td>93</td>
<td>36</td>
</tr>
<tr>
<td><strong>TnA</strong></td>
<td>Control: 10</td>
<td>100</td>
<td>20</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td>STI: 40</td>
<td>100</td>
<td>67</td>
<td>40</td>
</tr>
<tr>
<td><strong>Cb</strong></td>
<td>Control: 0</td>
<td>91</td>
<td>0</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>STI: 13</td>
<td>100</td>
<td>50</td>
<td>36</td>
</tr>
</tbody>
</table>

Table 3. Percentage of samples with detectable steroids levels in juvenile males who experienced a control condition or simulated territorial intrusion (STI). Steroids were considered detectable (bolded) when \( \geq 40\% \) of samples in a group were detected by LC-MS/MS.
3.2.1 Progesterone

In the blood, progesterone tended to increase ($W = 45, p = 0.0537$) in juveniles who experienced an STI (Figure 6). Plasma concentrations of progesterone were significantly increased by an STI ($W = 44, p = 0.0473$) and were approximately two-fold higher than blood concentrations, as expected (Jalabert et al., 2021). Further, blood progesterone levels were approximately eight-fold higher than brain progesterone levels in STI subjects. For brain progesterone levels, a mixed-effects ANOVA with Benjamini-Hochberg correction revealed a significant main effect of STI ($F(1, 23) = 52.787, p < 0.0001$) and region ($F(10, 230) = 27.220, p < 0.0001$; Figure 6B). Furthermore, there was a significant interaction between STI and brain region ($F(10, 230) = 27.220, p < 0.0001$). Post-hoc analyses revealed an increase in progesterone levels after an STI in the VMH, VTA, CG, and TnA (all $p < 0.0001$). Brain progesterone was non-detectable in control subjects.

Further analyses revealed that, among STI juveniles, blood progesterone levels did not correlate with brain progesterone levels (Figure 6C). However, there were positive correlations among the VMH, VTA, CG, and TnA. Neither blood nor brain progesterone levels correlated with behaviour in STI subjects.
Figure 6. Progesterone levels in non-breeding juvenile male song sparrows in (A) blood and (B) brain. (C) Matrices of Spearman correlations with Benjamini-Hochberg correction showing the correlations in progesterone levels between brain regions and blood in STI subjects. Significant correlations are shown, where red indicates a positive correlation. Juvenile Control n = 11, Juvenile STI = 15. Data are mean ± SEM. nd = non-detectable. # p = 0.0537, *** p < 0.0001.
3.2.2 Glucocorticoids

Corticosterone was significantly elevated in the blood of juveniles exposed to an STI (W = 7, p < 0.0001; Figure 7A). Plasma corticosterone levels were approximately two-fold higher than blood levels in both groups and plasma corticosterone also increased after an STI (W = 20, p < 0.01). Blood corticosterone levels exceeded brain levels in both conditions (Figure 7A). For brain corticosterone levels, there were significant main effects of STI (F(1, 22) = 33.645, p < 0.0001) and region (F(4.63, 101.91) = 32.083, p < 0.0001) and a significant interaction between the effect of STI and region (F(4.63, 101.91) = 3.889, p < 0.01; Figure 7B). Post-hoc analyses showed that, in all brain regions examined, brain corticosterone levels were significantly elevated by STI (all p < 0.01).

Furthermore, Spearman correlations with Benjamini-Hochberg correction showed that, among STI juveniles, brain corticosterone levels were strongly positively correlated between regions (Figure 7C). Moreover, blood corticosterone levels positively correlated with corticosterone levels in the POA, AH, VMH, NCM, and VTA. However, corticosterone in blood and brain did not correlate with behaviour in STI subjects.
Figure 7. Corticosterone levels in non-breeding juvenile male song sparrows in (A) blood and (B) brain. (C) Matrices of Spearman correlations with Benjamini-Hochberg correction showing the correlations in corticosterone levels between brain regions and blood in STI subjects. Significant correlations are shown, where red indicates a positive correlation. Juvenile Control n = 11, Juvenile STI = 15. Data are mean ± SEM. nd = non-detectable. *p < 0.05.
Like corticosterone, DHC was also significantly elevated in the blood after an STI (W = 5, p < 0.0001; Figure 8A). Plasma DHC levels were approximately two-fold higher than blood DHC levels in both groups. Blood DHC levels exceeded brain DHC levels. For brain DHC, there were significant main effects of STI (F(1, 22) = 45.032, p < 0.0001) and region (F(4.61, 101.51) = 50.756, p < 0.0001; Figure 8B). There was also a significant interaction between STI and region on DHC levels (F(4.61, 101.51) = 6.256, p < 0.0001). Post-hoc analyses revealed selective increases in DHC levels in the AH, BnST, Cb, TnA, and VMH (all p < 0.0001) along with a trending increase in the VTA (p = 0.0567) after an STI.

Unlike corticosterone, brain DHC levels did not significantly correlate with blood DHC levels in STI juveniles. Furthermore, there were very few significant correlations among brain regions. The AH and VMH, LS and NCM, VMH and VTA, NCM and Cb, and VTA and Cb positively correlated with each other (Figure 8C). Blood and brain DHC levels did not significantly correlate with behaviour in STI subjects.
Figure 8. DHC levels in non-breeding juvenile male song sparrows in (A) blood and (B) brain. (C) Matrices of Spearman correlations with Benjamini-Hochberg correction showing the correlations in DHC levels between brain regions and blood in STI subjects. Significant correlations are shown, where red indicates a positive correlation. Juvenile Control n = 11, Juvenile STI = 15. Data are mean ± SEM. nd = non-detectable. # p = 0.0567, *** p < 0.0001.
3.2.3 ANDROGENS

Testosterone, 5α-DHT, androstenedione, and DHEA were non-detectable in blood and plasma. Similarly, brain levels of androgens were non-detectable except for testosterone which was only detectable in the VTA and the TnA. Testosterone was detectable in 50% of control subjects and 21% of STI subjects in the VTA (thus, considered non-detectable in the STI group; Table 3). In the TnA, testosterone was detectable in 40% of the STI subjects and in 30% of control subjects (thus, considered non-detectable in the control group; Table 3). For brain testosterone, there was no effect of STI (F(1, 22) = 1.473, p = 0.2380), but there was a significant effect of region (F(10, 220) = 3.019, p < 0.001) and a significant interaction between STI and region (F(10,220) = 3.363, p < 0.001). Post-hoc analyses revealed that only in the TnA was testosterone significantly elevated after an STI (p < 0.0001).

3.2.4 ESTROGENS

E₂, 17α-estradiol, estrone, and estriol were non-detectable in blood, plasma, and brain.
4. DISCUSSION

In the current study, we measured aggressive behaviour and steroids in the blood and brain of wild non-breeding juvenile male song sparrows exposed to a territorial challenge or control condition. Like non-breeding adult males, juveniles who experience an STI are robustly aggressive compared to controls. In circulation and in the brain, progesterone, corticosterone, and DHC are elevated in STI subjects. However, androgens and estrogens are non-detectable or near non-detectable in blood and brain regardless of social context. Juvenile aggression might therefore be modulated by adrenal-derived or brain-derived steroids. Unlike adults, juvenile aggression seems to rely on glucocorticoids and progestogens rather than androgens and estrogens.

4.1 BEHAVIOUR

4.1.1 EFFECT OF STI ON JUVENILE AGGRESSION

Juveniles exposed to a 10-minute STI respond earlier, sing, fly, and spend more time near the decoy intruder compared to controls. These data agree with previous studies on adult male aggression during the breeding and non-breeding seasons (Heimovics et al., 2016; Pradhan et al., 2010; Soma et al., 2002). Control juveniles rarely fly, vocalize (i.e., call, song), or approach the empty cage within 5 meters. This aligns with non-breeding adult aggression in which controls are also minimally responsive (Newman & Soma, 2011). Aggressive displays are energetically costly. During the non-breeding season, it is beneficial to avoid agonistic encounters when there are no immediate fitness benefits (i.e. mate acquisition) and energy conservation is critical for survival (Arcese, 1989a, 1989b; Wingfield et al., 2001). As such, spontaneous displays of aggression are not expected and rarely observed in control subjects.
4.1.2 Juvenile Versus Adult Aggression

Juveniles exposed to an STI respond, sing, and fly at comparable levels compared to non-breeding adults. However, STI juveniles spend more time near the decoy intruder compared to their adult counterparts. Time spent within 5 meters is one of the most reliable indicators of overall aggression compared to flights or songs, which can be more variable across individuals (Wingfield, 1985; Wingfield & Wada, 1989). Spontaneous aggressive displays in control subjects does not differ by age.

Some previous reports indicate that juveniles can be more aggressive and responsive to playback than non-breeding adults. This is particularly true during late molt and in the early non-breeding season (Nordby, 1999; Wingfield & Hahn, 1994; Wingfield & Soma, 2002). Younger males (1 or 2 years) tend to enter molt earlier than older adults probably because older adults have more broods during late summer, which could delay molt (Dhondt & Smith, 1980; Hudson et al., 2008). The delay between younger and older males in completing molt could provide juvenile floaters, who have yet to establish their own territory, a chance to gain control of a territory. This can be achieved by aggressively intruding on another’s territory or by passively taking over a vacant territory (Arcese, 1989a; Hiebert et al., 1989). During late breeding and early autumn, juvenile male song sparrows are chased out of territories less frequently than adult intruders (Templeton et al., 2012b). This is likely because the energy expended chasing away a juvenile floater is more costly than the presence of the floater itself, especially if the adult resident has not yet completed molt. For juvenile floaters, it is beneficial to establish a territory at this life stage. Juvenile territory owners are more likely to survive and reproduce compared to juvenile floaters (Arcese, 1989b). Juveniles who successfully establish a territory before their
first breeding season are also more likely to own a territory as an adult (Arcese, 1989a). Thus, juvenile floaters have the opportunity (tolerance by adults) and the motivation (fitness benefits) to engage in agonistic encounters for territory control.

4.2 Steroids in Blood and Brain

In non-breeding juvenile male song sparrows, progesterone, corticosterone, and DHC are detectable in blood and brain. Apart from testosterone in the VTA and TnA, androgens and estrogens are non-detectable regardless of social context. Overall, we observe an increase in circulating steroids in subjects exposed to an aggressive encounter. In blood, progesterone tends to increase after an STI and glucocorticoids are significantly elevated after an STI. Progesterone, corticosterone, and DHC, which are all produced in the adrenal glands, are higher in blood compared to brain. In the brain, we observe region-specific elevation of progesterone and DHC and general elevation of corticosterone in all regions after an STI. Despite steroid elevation in the periphery and central nervous system, blood and brain progesterone are not correlated. This is also true for DHC. However, blood and brain corticosterone are positively correlated, specifically between blood and the POA, AH, VMH, NCM and VTA.

4.2.1 Progesterone

In juvenile male song sparrows, brain progesterone levels are rapidly modulated by an aggressive social interaction in a region-specific manner. After an STI, progesterone selectively increases in the VMH, VTA, CG, and TnA with non-detectable levels in other regions. This region-specific selectivity is in sharp contrast with control subjects who have non-detectable levels of progesterone throughout the brain. Unlike in the brain, blood progesterone is detectable
in both control and STI subjects, and there was a trending increase in blood progesterone in response to an STI. In non-breeding adult male song sparrows exposed to a 10-minute STI, progesterone is elevated in the blood and locally upregulated in the VTA and TnA, like in juvenile males (Jalabert et al., unpublished work). Interestingly, non-breeding adult males show no change in progesterone levels in the CG after a 10-min STI, unlike in juvenile males (Jalabert et al., unpublished work). Also, in breeding adult male song sparrows exposed to an STI, there are no changes in blood progesterone nor in brain progesterone (Jalabert et al., unpublished work). Similarly, breeding white-crown sparrows show no changes in circulating progesterone after an STI (Charlier et al., 2011). However, breeding male rufous horneros (Furnarius rufus), an ovenbird in South America, show an increase in plasma progesterone, but not testosterone, after an STI. In female song sparrows, there is no effect of STI on plasma progesterone during the breeding nor non-breeding seasons (Elekonich & Wingfield, 2000). Interestingly, female mice exposed to an aggressive interaction show a reduction in circulating progesterone (Davis & Marler, 2003). These seemingly contradictory results suggest that the effect of an aggressive social interaction on progesterone might be season- and sex-specific.

Progesterone might be locally synthesized within the juvenile avian brain during or after an aggressive encounter. Mechanistically, there is strong evidence to support this hypothesis. Progesterone levels in the blood do not correlate with levels in the VMH, VTA, CG, or TnA. This indicates that it is unlikely that progesterone is passively transported from circulation into the brain. Moreover, CYP11A1, which converts cholesterol into pregnenolone, and 3β-HSD, which converts pregnenolone into progesterone, are highly expressed in the developing and adult zebra finch brain (London et al., 2003, 2006). Activity of brain 3β-HSD is highest during the non-breeding season, at least in adult male song sparrows (Pradhan & Soma, 2012). After an
STI, 3β-HSD activity is also rapidly upregulated in the central medial and caudal telencephalon, which include the BnST, LS, and TnA (Pradhan et al., 2010). The season- and context-dependent upregulation of brain 3β-HSD activity could certainly support the conversion of pregnenolone into progesterone to facilitate non-breeding aggression in juveniles. In agreement with Pradhan et al., 2010, we observe rapid increases in brain progesterone in the TnA after an STI. Given the region-specific upregulation of progesterone after an STI, the dissociation between peripheral and central progesterone levels, and the rapid upregulation of brain 3β-HSD activity after an STI, it is likely that progesterone is locally synthesized in the juvenile brain either de novo from cholesterol or from circulating pregnenolone.

Progesterone might modulate non-breeding aggression directly or indirectly through different mechanisms. Progesterone might bind to progesterone receptors throughout the SDMN to directly modulate aggression. Like other steroid receptors, progesterone receptors are widely expressed throughout the avian brain with particularly high expression in the hypothalamus, POA, BnST, thalamus, and hippocampus (reviewed in Diotel et al., 2018; Gahr, 2001). Progesterone can also be metabolized into other active steroids to influence behaviour. Allopregnanolone is an active neurosteroid and metabolite of progesterone that positively modulates GABA signalling and is neuroprotective (Diviccaro et al., 2022; Wang et al., 2008). Allopregnanolone also influences aggression in rodents by acting on GABA\textsubscript{A} receptors (Soma et al., 2008). Diminished allopregnanolone levels are associated with elevated aggressive displays in mice (Pinna et al., 2005, 2008). However, at moderate doses, intraperitoneal injections of allopregnanolone increase the motivation to engage in aggressive challenges and the number of aggressive displays during a five-minute territorial challenge (Fish et al., 2002).
In addition to allopregnanolone, progesterone can also be converted into androstenedione via CYP17A1. Androstenedione is an active androgen that binds with moderate affinity to androgen receptors (Pradhan et al., 2010). Additionally, androstenedione is a precursor to testosterone and estrogens (Figure 1) which are both implicated in modulating aggression in adult male song sparrows. However, androstenedione is non-detectable in the blood and brain of juveniles and non-breeding adults (Jalabert et al., 2021). It is possible that androstenedione is synthesized from progesterone and rapidly metabolized into other androgens and estrogens to support aggression and is therefore non-detectable in the current study. Overall, more work is necessary to directly investigate progesterone and its metabolites as potential modulatory mechanisms of non-breeding aggression in juvenile male song sparrows.

4.2.2 GLUCOCORTICOIDS

Stressful social interactions such as an agonistic encounter can have rapid and profound effects on corticosterone levels in the blood and brain. In non-breeding juvenile male song sparrows, corticosterone is rapidly upregulated in all brain regions investigated and is positively correlated with blood corticosterone levels. As observed in the current study, non-breeding adult male song sparrows exposed to a 10-minute STI show rapid upregulation of corticosterone in the blood and the brain compared to controls (Jalabert et al., unpublished data). In contrast, non-breeding adult males exposed to a 30-minute STI show no changes in blood nor brain corticosterone levels (Newman & Soma, 2011). This indicates that the duration of social stimuli exposure differentially affects corticosterone synthesis and metabolism. Breeding adult males exposed to a 30-minute STI show a significant upregulation of blood and brain corticosterone levels (Newman & Soma, 2011). Similarly, white-crowned sparrows and great tits (Parus major)
exposed to an STI also demonstrate an increase in plasma corticosterone during breeding (Charlier et al., 2011; Van Duyse et al., 2004). These data suggest that, like in non-breeding adult males, juveniles exposed to a 10-minute STI might rapidly synthesize corticosterone in the adrenal glands before corticosterone is transported through circulation into the brain.

After rapid synthesis in the adrenals, corticosterone is transported to the brain to facilitate a stress response. This hypothesis aligns with previous reports that found a trending increase in plasma corticosteroid-binding globulin (CBG) concentrations after a 30-minute STI in breeding adult male white-crowned sparrows (Charlier et al., 2011). CBG is a hepatic carrier protein responsible for transporting progestogens and glucocorticoids in circulation (Lin et al., 2021). Interestingly, birds lack sex hormone binding globulin and CBG is the primary carrier of androgens in addition to progestogens and glucocorticoids (Lin et al., 2021; Rensel & Schlinger, 2016; Vashchenko et al., 2016). Once corticosterone enters the brain, it can bind to intracellular mineralocorticoid receptors and glucocorticoid receptors and/or on membrane-bound glucocorticoid receptors, which are highly expressed throughout the songbird brain (Rensel et al., 2018; Rensel & Schlinger, 2016). There, corticosterone can have profound rapid (via membrane-bound receptors) and enduring (via intracellular receptors) effects on physiology and behaviour.

Like corticosterone, DHC is upregulated in the blood and brain of non-breeding STI juvenile male song sparrows. DHC is an inactive metabolite of corticosterone that has received much less attention in the literature compared to corticosterone. Unlike corticosterone which shows global elevation in the brain, DHC is elevated only in the AH, BnST, TnA, and Cb after an STI. Moreover, DHC concentrations do not highly correlate across regions nor with blood levels. Thus, DHC might be locally synthesized in the brain to help fine-tune the effects of
corticosterone on behaviour. Corticosterone can be inactivated into DHC by 11β-HSD2. 11β-HSD2 is expressed in the non-breeding zebra finch brain, particularly in the Cb, diencephalon (contains AH, VMH), optic tectum, NCM, hippocampus, and caudal nidopallium (Rensel et al., 2018). Interestingly, expression of 11β-HSD1, which is responsible for the regeneration of corticosterone from DHC, is not detected in the non-breeding zebra finch brain (Rensel et al., 2018). Because we observe global upregulation of brain corticosterone but region-specific upregulation of brain DHC, it is likely that systemic corticosterone enters the brain passively, but local glucocorticoid action is fine-tuned via 11β-HSD2. In control subjects, many brain regions had non-detectable levels of DHC. It is probable that DHC is present in these regions but below our limit of detection. Altogether, the current data and previous work indicate that the rapid effects of an aggressive social encounter on glucocorticoids are time-sensitive, with glucocorticoids showing rapid upregulation and subsequent metabolism or inactivation in a matter of minutes.

Like progesterone, the relationship between aggression and glucocorticoids is complex and probably time-scale dependent. While there is an established link between chronic glucocorticoid elevation and diminished aggression, acute glucocorticoid elevation increases aggressive displays (Haller et al., 1998; Soma et al., 2008). In breeding adult male song sparrows, chronic corticosterone treatment diminishes aggression in subjects exposed to an STI (Wingfield & Silverin, 1986). In rodent models, the acute effects of glucocorticoids on aggression have also been investigated. Siberian hamsters (*Phodopus sungorus*) are more aggressive in non-breeding conditions (short day length and elevated melatonin) than in breeding conditions and this effect is attenuated when subjects are adrenalectomized (Demas et al., 2004). Importantly, subjects that receive adrenal demedullation, which prevents catecholamine
synthesis but not glucocorticoid synthesis, maintain non-breeding aggression (Demas et al., 2004). In adult golden hamsters (Mesocricetus auratus) in long day length conditions, microinjection of cortisol (homolog of corticosterone) directly into the AH rapidly increases aggression towards a submissive intruder (Hayden-Hixson & Ferris, 1991). In contrast, microinjection of testosterone, 17β-estradiol, and progesterone into the same brain region did not increase aggression (Hayden-Hixson & Ferris, 1991). Similarly, adult male rats acutely treated with metyrapone, which inhibits synthesis of corticosterone, show a rapid reduction in aggressive displays (Mikics et al., 2004). This effect is rescued by systemic corticosterone administered just two minutes before behavioural testing (under 10 minutes overall; Mikics et al., 2004), indicating that glucocorticoids can rapidly modulate aggressive behaviour. Furthermore, glucocorticoids and repeated exposure to stressful social contexts accelerate development of adult-like aggressive responding in juvenile golden hamsters (Wommack et al., 2005).

The rapid effects of glucocorticoids on aggression are presumably mediated by non-genomic secondary messenger signalling cascades and interactions with other endocrine mechanisms (Haller et al., 1998). Corticosterone can help release bound, inactive testosterone into its free and active state which, in turn, can support metabolically challenging behaviours like agonistic interactions (Orchinik et al., 2002). Furthermore, glucocorticoids are in part responsible for facilitating energy mobilization and gluconeogenesis during energetically costly interactions, such as during an STI (Landys et al., 2006; Sapolsky et al., 2000). Importantly, after aggressive interactions have terminated and a winner and loser have been established, circulating glucocorticoids rapidly decrease in winners but not losers (Haller et al., 1998). This distinction in the timescale of glucocorticoid action, which goes beyond the scope of the present study, indicates that glucocorticoid action can be adaptive in the short-term to facilitate energy
mobilization so an individual can win an agonistic encounter. However, for losers, it may be equally as adaptive to maintain elevated glucocorticoid levels because this might help the individual remain alert for a secondary encounter. If chronically elevated, glucocorticoids in losers of agonistic encounters can also diminish aggressive responsiveness. In turn, this would decrease the chances of losers engaging in future fights that could put the weaker individual at risk of physical harm. Altogether, the role of circulating and neural glucocorticoids on non-breeding juvenile aggression is likely time- and context-dependent.

4.2.3 ANDROGENS

In non-breeding juvenile male song sparrows, testosterone, like the other androgens investigated, was non-detectable in blood and non-detectable or near non-detectable in the brain. After an STI, testosterone is elevated only in the TnA. However, 60% of samples are non-detectable in STI subjects and 70% of samples are non-detectable in controls (Table 3). Similarly, non-breeding adult male song sparrows have non-detectable or near non-detectable levels of androgens in circulation regardless of social context (Jalabert et al., 2021; Jalabert et al., unpublished data). In the brain, non-breeding adult males exposed to an STI show region-specific changes in testosterone levels in the POA, AH, TnA and NAc (Jalabert et al., unpublished data). While brain testosterone might support non-breeding adult aggression, the current findings suggest that non-breeding juvenile aggression is likely decoupled from the modulatory effects of androgens.
4.2.4 Estrogens

Estrogens are non-detectable in the blood and brain of non-breeding juvenile male song sparrows regardless of social context. Free-living non-breeding adult males also have non-detectable levels of estrogens in blood and brain (Jalabert et al., unpublished data). In captive non-breeding adult male song sparrows, E₂ is detectable but unaffected by STI in plasma and in the brain (Heimovics et al., 2016). In breeding adult males, estrogens are non-detectable in blood and detectable but unaffected by STI in the brain (Jalabert et al., unpublished data). Male white-crowned sparrows exposed to a 15-minute or 30-minute STI during late breeding show no change in plasma E₂ but an elevation of E₂ in certain brain regions (Charlier et al., 2011).

While the effects of an aggressive social interaction on estrogen levels seem to be minimal, estrogens are implicated in supporting non-breeding aggression. Castration and androgen receptor antagonism do not affect non-breeding adult aggression (Sperry et al., 2010; Wingfield, 1994) but aromatase inhibition decreases non-breeding aggression (Soma et al., 2000a, 2000b). Furthermore, E₂ replacement rescues the effects of aromatase inhibition (Soma et al., 2000a). In juveniles, we did not find evidence to suggest peripheral nor central modulation of non-breeding aggression by estrogens, although a more sensitive technique might be necessary to detect the low endogenous levels at which estrogens typically exert their actions. Overall, adrenal-derived or brain-derived glucocorticoids and progesterone may compensate for the negligible levels of androgens and non-detectable levels of estrogens in sexually immature juveniles.
5. CONCLUSION

5.1 MAJOR FINDINGS AND IMPLICATIONS

The present data show that non-breeding aggression in juvenile male song sparrows is robust. Juvenile males are as aggressive or even more aggressive than their non-breeding adult counterparts. An investigation of the steroid milieu in blood and brain by LC-MS/MS reveals that the endocrine mechanisms that might support juvenile aggression are distinct from those in adults. In blood, there is a trending increase in progesterone and a significant increase in glucocorticoids in subjects exposed to an aggressive social interaction. In the brain, progesterone and DHC are upregulated in a region-specific manner while corticosterone is globally upregulated in all regions after an aggressive social interaction. Androgens and estrogens are non-detectable or near non-detectable in blood and brain regardless of social context. Juvenile aggression might therefore be independent of androgens and estrogens and instead be modulated by adrenal-derived or brain-derived progesterone and glucocorticoids. To our knowledge, this study is the first to investigate the neuroendocrine mechanisms of non-breeding aggression in a juvenile avian model. This work provides fundamental insights into the potential role of neural progestogens and glucocorticoids in modulating juvenile aggression.

5.2 LIMITATIONS AND FUTURE DIRECTIONS

We observe region-specific elevation of brain progesterone and DHC including nodes of the SDMN, while other regions show no changes or have non-detectable steroid levels. This suggests that there is region-specific neuroendocrine modulation of aggression in juvenile males. However, brain steroid analyses were conducted with a limited amount of tissue (0.245 to
1.47mg) and steroids can have profound physiological and behavioural effects at extremely low concentrations. Given these considerations, it is possible that some samples might still contain physiologically relevant steroid levels but fall below our limit of detection. Novel derivatization methods could help attain lower detection limits and offer one possible solution to this limitation.

In conjunction with low amounts of tissue in samples, non-detectable samples are an obstacle for statistical analyses. As mentioned above, samples that are considered non-detectable can still contain steroids somewhere between zero and the limit of detection. Some researchers have suggested that non-detectable samples should be replaced either with zero, the detection limit, the detection limit divided by 2, or the detection limit divided by $\sqrt{2}$ (Handelsman & Ly, 2019). Others have created a method to accurately impute non-detectable values for biological samples that fall below the detection limit (Wei et al., 2018a, 2018b). Here, we used the METIMP tool to impute steroid values in groups that had at least 40% detectable samples, similar to previous studies and as recommended by the creators of METIMP (Hamden et al., 2021; Jalabert et al., 2021; Wei et al., 2018b). For groups that did not meet the 40% detectable criteria, non-detectable values were replaced with zeros to conduct statistical analyses. With progesterone, many brain regions failed to meet the 40% detectable criteria and were therefore replaced with zeros, which greatly inflated the number of zeros in the dataset. While log-transforming data helped ameliorate violations of homogeneity of variances, there remains a slightly increased chance of attaining a type I error by performing parametric analyses on the progesterone dataset. In most cases, as observed with the glucocorticoids, very few regions failed to reach this criterion and statistical analyses using log-transformed data were appropriate.

The effects of an acute (10-minute) aggressive interaction on blood and brain steroid profiles in juvenile male song sparrows are rapid and profound. However, because steroid
content was not directly manipulated, we cannot know if progestogens and glucocorticoids are upregulated to support aggressive responsiveness. Future studies should address this limitation by performing a direct manipulation of steroid concentrations and observing behavioural outcomes. Critically, there have been contradictory reports of the promoting or inhibiting effects of these steroids which is likely due to temporal differences in steroid action (reviewed in Soma et al., 2008). Therefore, future work should investigate the temporal effects of progestogens and glucocorticoids on aggression by using chronic (days to weeks) steroid implant manipulations and acute (minutes) steroid receptor antagonist manipulations.

The rapid elevation of brain progesterone observed in the current study is a novel finding, particularly in the context of juvenile non-breeding aggression. Previous studies that investigated the role of progesterone in modulating aggression have primarily done so under breeding conditions and have only looked at circulating progesterone (Adreani et al., 2018; Davis & Marler, 2003). In female song sparrows, plasma progesterone is not affected by STI in the breeding nor non-breeding seasons (Elekonich & Wingfield, 2000). However, in adult males exposed to an STI, blood and brain progesterone is upregulated in the non-breeding season (Jalabert et al, unpublished data). Future work should follow-up on the current findings and determine the sex-specific role, if any, of brain progesterone in modulating non-breeding aggression in male song sparrows.

Like progesterone, the effects of glucocorticoids on aggression are nuanced and likely time-scale dependent. While this study is one of the first to quantify active and inactive glucocorticoids in discrete brain regions of a juvenile songbird under different social contexts, the source of corticosterone and DHC synthesis and action remains unclear. While enzyme expression studies in other songbird models point to activation and inactivation of
glucocorticoids as a regulatory mechanism of glucocorticoid action (Rensel et al., 2018; Rensel & Schlinger, 2016), this goes beyond the scope of the current study. Future studies should investigate enzyme expression—and more importantly, enzyme activity—in discrete brain regions under different seasonal and social contexts. Furthermore, few studies have examined changes in avian glucocorticoid and mineralocorticoid receptor expression under different seasonal and social contexts (Breuner & Orchinik, 2001). This, in conjunction with a closer investigation of CBG concentrations under the same conditions, warrants investigation at distinct developmental life stages.

Here, we provide novel insights into the endocrine and neuroendocrine modulation of social behaviour at a juvenile life stage. Sex-specific modulatory mechanisms of aggression are beyond the scope of this study but still warrant further investigation. Female song sparrows, like juvenile males, are aggressive (Arcese et al., 1988; Elekonich & Wingfield, 2000) and rapid neurosteroid changes have been observed in response to socially relevant stimuli in the female songbird brain (de Bournonville et al., 2020). Together with the above-mentioned sex-specific effects of progesterone on aggression, these data implore future work to incorporate females in subsequent studies.

5.3 CONCLUSIONS

Juvenile avian aggression is robust and seems to be independent of androgens and estrogens, which differs from the endocrine mechanisms that support adult avian aggression. In sexually immature juveniles, non-breeding aggression may instead be supported by adrenal-derived and brain-derived progesterone and glucocorticoids. This work provides novel insights into the modulatory role of neurosteroids on social behaviours in juvenile birds. Because steroid
signaling pathways are highly evolutionarily conserved, this could have important implications for the neuroendocrine modulation of aggression in other vertebrates and model systems
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