NEUROENDOCRINE MODULATION OF AGGRESSIVE BEHAVIOR

by

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Abstract

Aggression is an important social behavior that plays key roles in mediating access to limited resources. During the breeding season, aggression is stimulated by gonadal steroids. Interestingly, many species are highly aggressive during the non-breeding season, despite the gonads being regressed. Song sparrows, *Melospiza melodia*, are territorial year-round and therefore are an excellent animal model to study seasonal changes in the steroid modulation of aggression. Recent research has shown that the brain itself can produce steroids by metabolizing circulating precursors and even by *de novo* synthesis from cholesterol. Because steroids can be synthesized locally in the brain, steroid levels in the blood often do not reflect steroid levels in specific brain regions. Thus, it is critical to accurately measure steroid levels in discrete brain regions. Steroids are challenging to measure because they are present at very low levels and current techniques often lack the sensitivity required. In chapter 2, I developed and validated a liquid chromatography-tandem mass spectrometry assay for the measurement of steroids with a focus on androgens and their precursors. In chapter 3, I developed and validated a method to measure several estrogens with increased sensitivity. Both methods were validated in blood, plasma, and microdissected brain tissue of song sparrows and are broadly applicable to other species, allowing steroid profiling in circulation and microdissected brain. Further, we applied these methods and quantified steroids examining peripheral and neural synthesis of steroids across seasons and in response to an aggressive interaction in wild male song sparrows. Briefly, I report that 1) brain steroid levels can greatly differ from circulating steroid levels, 2) brain steroid levels show region-specific seasonal patterns that are not a simple reflection of circulating steroid levels, and 3) local steroid production rapidly increases in response to an
aggressive interaction in the non-breeding season. Overall, steroid levels are regulated within the brain and local production is dependent on the season and behavioral context.
Lay Summary

Steroid hormones are important modulators of the brain and behavior. They are secreted by glands such as gonads and adrenals into the bloodstream to exert their actions in target tissues. In addition, the brain itself can locally produce steroids, but far less is known about local steroid production in the brain. In this dissertation, I present a series of studies examining peripheral and neural synthesis of steroids across seasons and in response to a social aggressive interaction in wild male song sparrows. Briefly, I report that 1) brain steroid levels can greatly differ from circulating steroid levels, 2) brain steroid levels show region-specific seasonal patterns that are not a simple reflection of circulating steroid levels, and 3) local steroid production in the brain rapidly increases in response to a social aggressive interaction in the non-breeding season. Overall, steroid levels are regulated within the brain and local production is dependent on the season and behavioral context.
Preface

All projects and methods were approved by the University of British Columbia’s Animal Care Committee (Certificate number A16-0234).


A version of Chapter 2 has been published. Jalabert, C., Ma, C., and Soma, K.K. (2021) Profiling of systemic and brain steroids in male songbirds: Seasonal changes in neurosteroids. Journal of Neuroendocrinology. 33 (1): e12922. I was the lead investigator, responsible for all major areas of concept formation, data collection and analysis, as well as manuscript publication. C. Ma assisted in the development of the protocols. K. Soma was the supervisory author on this project and was involved throughout the project in concept and manuscript composition.

A version of Chapter 3 has been published. Jalabert, C., Shock, M.A., Ma, C., Bootsma, T.J., Liu, M.Q., and Soma, K.K. (2022) Ultrasensitive quantification of multiple estrogens in songbird blood and microdissected brain by LC-MS/MS. eNeuro. 9 (4). I was the lead investigator, responsible for all major areas of concept formation, data collection and analysis, as well as preparation of the manuscript for publication. M. Shock and T. Bootsma assisted in collection of the tissue samples. M. Shock, C. Ma, T. Bootsma, and M. Liu assisted in the
development the protocols. K. Soma was the supervisory author on this project and was involved throughout the project in concept and manuscript composition.

The study described in chapter 4 has not been submitted for publication as of the thesis submission date. For this project, I was the lead investigator responsible for all major areas of concept formation as well as data collection and analysis, supervised by K. Soma throughout the project.
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List of Abbreviations

17α-E2 – 17α-estradiol
17β-E2 – 17β-estradiol
17β-HSD – 17β-hydroxysteroid dehydrogenase
2Me-E2 – 2-methoxyestradiol
2OH-E2 – 2-hydroxyestradiol
3β-HSD – 3β-hydroxysteroid dehydrogenase/Δ5-Δ4 isomerase
4Me-E2 – 4-methoxyestradiol
4OH-E2 – 4-hydroxyestradiol
5α-DHT – 5α-dihydrotestosterone
ACN – acetonitrile
AH – anterior hypothalamus
ANOVA – analysis of variance
AR – androgen receptors
BnST – bed nucleus of the stria terminalis
Cb – cerebellum
CBG – corticosteroid binding globulin
CG – central grey
CIHR – Canadian Institute of Health Research
cm – centimeter
CoA – anterior commissure
COMT – catechol O-methyl transferase
CV – coefficient of variation
CYP17A1 – 17α-hydroxylase/17,20-lyase
CYP1A1 – estrogen-2-hydroxylase
DHEA – dehydroepiandrosterone
DMIS – 1,2-dimethylimidazole-5-sulfonyl chloride
DSCL – dansyl chloride
E₁ – estrone
E₃ – estriol
ER – estrogen receptors
FMP-TS – 2-fluoro-1-methylpyridinium-p-toluenesulfonate
g – gram
GPER-1 – G-protein-coupled estrogen receptor-1
h, hr – hours
HPLC – High Performance Liquid Chromatography
IS – internal standard
ISCL – 1-methylimidazole-2-sulfonyl chloride
LC – liquid chromatography
LC-MS/MS – liquid chromatograph tandem mass spectrometry
LLOQ – lower limit of quantification
LS – lateral septum
M – Molar
m/s – meters per second
m/z – mass/charge ratio
MeOH – methanol
mg – milligram
min – minutes
ml, mL – milliliter
mm – millimeter
MPA – mobile phase A
MPB – mobile phase B
MPPZ – methyl-1-(5-fluoro-2,4-dinitrophenyl)-4-methylpiperazine
MRM – multiple reaction monitoring
mRNA – messenger ribonucleic acid
mV – millivolts
n – sample size
na – not applicable
NAc – nucleus accumbens
NCM – caudomedial nidopallium
nd – nondetectable
ng – nanograms
ns – nonsignificant
NSERC – Natural Sciences and Engineering Research Council
ºC – degrees Celsius
PBS – phosphate buffered saline
pg – picograms
POA – preoptic area
PR – progesterone receptors
Q1 – quadrupole 1
QC – quality control
SBN – social behaviour network
SDMN – social decision-making network
sec – seconds
SEM – standard error of the mean
sMRM – scheduled multiple reaction monitoring
SPE – solid phase extraction
STI – simulated territorial intrusion
T – testosterone
TnA – nucleus taeniae
TrSM – septopalliomesencephalicus
UBC – University of British Columbia
uHPLC – ultra high performance liquid chromatography
VMH – ventromedial hypothalamus
VTA – ventral tegmental area
WB – whole blood
μg – microgram
μL – microliter
μm – micrometer
μM – micromolar
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Chapter 1: Introduction

1.1 Aggression

Aggression is one of the most important social behaviors. It is displayed by virtually all animals and serves a wide range of adaptive functions. Aggressive behaviors enable the acquisition and defense of limited valuable resources, such as mates, territories, shelters, and food (Austad, 1983). However, aggressive encounters are also a costly investment in terms of time, energy, predation risk, and physical injury. Animals evaluate the costs and benefits of competing for resources in search of maximal fitness payoffs to make the decision to fight or not fight. In this way, aggressive behaviors mediate the establishment of dominance and subordination relationships among competitors that enable access to a given limited resource (King, 1973). In the context of choosing a mate, for example, the contenders evaluate strength and establish dominance in front of a potential mate. In solitary or territorial species, aggression implies exclusive use of a resource, but in gregarious species, aggression sets relations of dominance and establishes hierarchies. Consequently, aggressive interactions have a direct impact on the survival and reproductive success of individuals.

Historically, studies on aggression have focused on breeding male-male competition. However, aggression is not exclusive to males and does not only occur during competition for a mate. Female-female contests, although less studied, are also seen in nature. Just like in males, preferential access to a resource is advantageous for females, which drives this behavior. Aggression has been defined in a variety of ways. Aggression has traditionally been defined as an overt behavior with the intention of inflicting physical damage upon another individual (Nelson, 2005). Different classifications of aggressive interactions have been proposed
(Wingfield et al., 2006), although they have only more recently been defined around the context in which they occur: 1- Territorial aggression; 2- Disputes over food; 3- Aggression to establish relationships of dominance; 4- Parental aggression; 5- Aggression for sexual competition; 6- Antipredator aggression; 7- Irritable aggression.

1.2 Neural control of aggression

The regulation of social behavior, such as aggression, depends on neural circuits, including the social behavior network (SBN) (Newman, 1999). This well-studied neural circuit consists of reciprocally connected brain regions, or nodes, located in the forebrain, midbrain, and hindbrain. In mammals, the SBN consists of six nodes: the extended medial amygdala [the medial amygdala and the medial bed nucleus of the stria terminalis (BnST)], the lateral septum (LS), the preoptic area (POA), the anterior hypothalamus (AH), the ventromedial hypothalamus (VMH), and the periaqueductal gray. The SBN receives and evaluates external stimuli, integrates them with internal physiological information, and produces an appropriate response with a distinctive circuit activity pattern, biasing behavior that is adaptive for a specific context. For example, across brain nodes, there is a particular pattern of neural activation for sexual behavior, and the same brain nodes show a different pattern of neural activation for aggressive behavior.

Social behavior emerged early in animal evolution, likely playing a key role in determining the survival and fitness of individuals. Therefore, its neural control is under strong evolutionary pressures. Neuroanatomical and functional studies that assessed the distribution, connectivity, and neurochemistry of the brain nodes of the SBN have found that the neural circuits that regulate social behavior are highly conserved across vertebrates and play similar roles in the regulation of these behaviors (Goodson, 2005). Similarly, despite the diversity of
social behaviors regulated by this network (i.e., aggression or paternal care), the neural circuits and hormones involved in modulating these behaviors have been highly conserved in all vertebrate lineages. Birds, reptiles, amphibians, and teleost fish all contain nodes of the SBN that are homologous with the mammalian counterparts described above and have similar activation patterns in similar social contexts. The conservation of these nodes enables comparative studies in different species to establish general principles among vertebrates (Goodson and Kabelik, 2009).

More recent work suggests that a broader social decision-making network (SDMN; Figure 1.1) regulates adaptive social behaviors in response to different context or stimuli (O’Connell and Hofmann, 2011). The SDMN consists of both the classic SBN nodes and also the mesocorticolimbic reward system. The mesocorticolimbic reward system is composed of several interconnected nodes, including the prefrontal cortex, ventral tegmental area (VTA), and nucleus accumbens (NAc), involved in the regulation of several different behaviors, including activational aspects of motivation, preparatory behavior, and appetitive behavior. The SDMN appeared early in evolution and is also highly conserved among vertebrates. Importantly, every node of the SBN and SDMN expresses sex steroid receptors, indicating that these brain regions are sex steroid-sensitive and that hormones play key roles in modulating the activity of these networks and regulating social behaviors.

1.3 Modulation of aggression in the breeding season

Traditionally, blood-borne gonadal steroids have been the primary focus of neuroendocrine studies on aggression. In seasonally-breeding vertebrates, the gonads grow before the breeding season and regress following the termination of breeding. Circulating sex
steroid concentrations fluctuate alongside gonadal growth and regression, and high levels generally occur during the breeding season and basal or nondetectable levels occur during the non-breeding season. The expression of aggressive behaviors occurs mostly in the reproductive context, mainly between males where aggressive behavior may be crucial to acquiring a mate and maintaining territorial boundaries. Aggression during the breeding season has been related to high levels of circulating sex steroid hormones such as testosterone (T) (Wingfield et al., 1990). This was shown mainly by two types of evidence. First, castration experiments, eliminating the main source of circulating T, result in a decrease or loss of aggression in different classes of vertebrates (and treatment with T restores aggression) (Vandenbergh, 1971; Balthazart, 1983; Moore and Marler, 1987; Wingfield and Hahn, 1994). Second, the temporal pattern of secretion of T is plastic and varies according to the social context. Aggressive interactions for 10 to 30 min can induce an increase in plasma T levels after the encounter, favoring the persistence of aggression against new stimuli (Wingfield et al., 1990, 2006). Numerous studies have provided evidence that circulating gonadal steroids, such as T and 17β-estradiol (17β-E2), promote aggression by binding directly to androgen receptors (AR) and estrogen receptors (ER) in the brain, modulating neural pathways relevant to aggressive behavior.

1.4 Modulation of aggression in the non-breeding season

While gonadal steroid secretion is important in modulating breeding aggressive behaviors, recent work indicates that these behaviors are not exclusively regulated by gonadal steroids. Some species of vertebrates exhibit year-round aggression, even outside of the breeding season, despite the fact that the gonads are regressed and circulating androgen levels are low.
High levels of aggression year-round may benefit individuals by allowing them to gain access to limited food resources and to defend their territories (Soma et al., 2008).

While the adaptive value of this alternative neuroendocrine mechanism of aggression is still being investigated, having high levels of T in circulation for prolonged periods of time may have major costs (e.g. suppression of immune function, increased metabolic rate). There are different mechanisms to maintain aggression and simultaneously avoid the costs of high circulating T during the non-breeding season. For example, utilizing inactive sex steroid precursors as dehydroepiandrosterone (DHEA) as a source of androgens could help in maintaining non-breeding aggression. Some animals that show elevated non-breeding aggression also display elevated plasma DHEA levels, suggesting that the adrenals and/or gonads are secreting DHEA into the circulation during the non-breeding season (Soma and Wingfield, 2001; Scotti et al., 2009). DHEA can travel in the circulation and be locally converted to active steroid hormones, such as T and 17β-E2, which can then bind with high affinity to AR and ER, respectively. Likewise, other DHEA metabolites (such as androstenediol) can modulate the expression of social behavior via conversion to T (Morali et al., 1974). There is now strong evidence that the brain expresses the appropriate steroidogenic enzymes capable of producing sex steroids, called neurosteroids, from inert precursors (such as DHEA) and even synthesizing these steroids de novo from cholesterol (Figure 1.2). The regulation of aggressive behaviors via this mechanism might be far less energetically costly than sustaining high levels of circulating sex steroids (Soma, 2006; Soma et al., 2008).
1.5 Slow and rapid steroid actions on brain

Sex steroids, such as androgens and estrogens, act on the central nervous system by modulating the activity of their target cells when they bind to their intracellular receptors (e.g., AR and ER). Bound receptors act as transcription factors to regulate gene expression in brain cells. This genomic mechanism of action allows steroid hormones to modulate the activity of the different SBN brain regions and act indirectly, but not deterministically, on the execution of a behavior (Adkins-Regan, 2005). For instance, sex steroids can change a stimulus response threshold, such as the response threshold toward a competitor, but do not typically trigger aggression itself. The genomic effects of steroid hormones on the central nervous system require several hours or days to develop and produce persistent changes in physiology and behavior (McCarthy, Wright and Schwarz, 2009).

In addition, sex steroids can exert short-term (within 30 min) non-genomic effects on behavior. For instance, 17β-E2 administration increases aggression within 15 min in short-day (winter-like) male mice, a time frame incompatible with a genomic mechanism (Trainor et al., 2007). The non-genomic effects of steroids are often mediated by plasma membrane receptors or by the allosteric modulation of neurotransmitter receptors. For example, ER, such as ERα and ERβ, can be associated with the plasma membrane and rapidly regulate intracellular signaling pathways (Micevych and Dominguez, 2009; Heimovics et al., 2012). Another membrane-associated ER is the G-protein-coupled estrogen receptor-1 (GPER-1), which is also present in the brain. Estrogens rapidly influence aggressive behaviors in birds and mammals via actions in the SBN and SDMN (Trainor and Nelson, 2012).
1.6 Steroid transport in blood

Steroids are transported in the blood by steroid-binding proteins. Generally, glucocorticoids and progestogens are transported by corticosteroid binding globulin (CBG), while androgens and estrogens are transported by sex hormone-binding globulin (SHBG). These transport proteins bind steroids with high affinity and specificity (Lin et al., 2021). However, in birds, SHBG has not been found (Wingfield, Matt and Farner, 1984), and recent work shows that androgens can also be transported by CBG, though with less binding affinity than the other steroids (Vashchenko et al., 2016). Albumin is another carrier protein that binds steroids with low specificity and affinity but is highly abundant in the circulation and is present in birds (Baker, 2002; Malisch and Breuner, 2010). Transport proteins also play important roles in steroid bioavailability. Steroid hormones must be released from their transport proteins to exert their actions in target tissues by either binding to membrane receptors or passively cross the cell membrane and bind to nuclear receptors. In this way, carrier proteins control the access of steroids to their target tissues (Mendel, 1989). Lastly, the vast majority of steroids (e.g. ~95% of corticosterone) are bound to these proteins, and interestingly, since unbound steroids are cleared more rapidly from the blood, it is proposed that these proteins may play a role as a steroid reservoir (Lin et al., 2021).

1.7 Neuroethological model: song sparrow

Despite the evolutionary divergence among vertebrates, the similar ecological problem faced by these organisms (the expression of aggressive behavior to defend a limited resource) may have led to a similar solution in terms of the underlying physiological mechanisms. In this sense, studies in different classes of vertebrates are especially important to shed light on common
issues and establish general principles. We have chosen songbirds since they are excellent models to study the relationship between hormones, their mechanisms of action, and aggressive behavior (Soma, 2006). First, they express high levels of steroidogenic enzymes in the brain (Schlinger and Remage-Healey, 2012). In particular song sparrows, *Melospiza melodia*, are an advantageous model for the study of the neuroendocrine bases of aggression. Territorial aggression is a behavior exhibited in wild song sparrows and shows natural changes throughout the annual cycle. This species shows aggression throughout the year (except during the molt), dependent on the gonads in the breeding season, and independent of the gonads in the non-breeding season, during which castration does not affect aggression (Wingfield, 1994). In this species, the levels of DHEA are elevated in plasma in the non-breeding season, and it is postulated that DHEA could be transformed to T and 17β-E2 locally in the brain (Newman et al., 2008; Soma & Wingfield, 2001). Recent studies have shown that 17β-E2 rapidly modulates aggressive behavior during the non-breeding season (Heimovics, Ferris and Soma, 2015). Photoperiod could be the environmental signal that influences the mechanism of action of steroids that act in the brain to regulate aggressive behavior. However, the mechanisms underlying these seasonal changes remain unclear.

As previously mentioned, neurosteroids can regulate behavior via actions on the SBN and SDMN. For example, in male song sparrows, aromatase, the enzyme that converts androgens into estrogens, is critical for the expression of aggression in the non-breeding season as aromatase inhibition decreases aggression even when circulating sex steroids are nondetectable (Soma *et al.*, 2000; Soma, Tramontin and Wingfield, 2000). Likewise, 17β-E2 administration increases aggression within 20 min in non-breeding males only (Heimovics, Ferris and Soma, 2015). Several brain regions, such as the POA, VMH, and BnST, contain elevated levels of
aromatase year-round. In contrast, aromatase activity decreases in the ventromedial telencephalon [which includes nucleus taeniae (TnA), the homolog to the mammalian medial amygdala], during the molt, when song sparrows also show a decrease in aggressive behaviors (Soma et al., 2003). Similarly, the activity of brain 3β-hydroxysteroid dehydrogenase/Δ5–Δ4 isomerase (3β-HSD), an enzyme that catalyzes the conversion of DHEA to an active androgen (Figure 1.2), changes with season and social context. In song sparrows, 3β-HSD activity is higher during the non-breeding season in multiple brain areas, and an aggressive social stimulus rapidly increases enzymatic activity in the brain (Pradhan et al., 2010). Together, these data provide evidence of neural steroidogenesis and suggest that local neurosteroid production modulates social behavior mainly during the non-breeding season.

Furthermore, aggressive encounters modulate DHEA brain levels in song sparrows. For instance, wild males were exposed to a simulated territorial intrusion (STI) (conspecific live decoy with a playback) for 30 min and then brachial vein (systemic steroids) and jugular vein (enriched in neurosteroids) levels of DHEA were measured. Breeding animals showed changes in both circulating and brain DHEA levels, whereas non-breeding males showed changes in only brain DHEA levels (Newman and Soma, 2011). Additionally, in captive song sparrows, steroid levels were measured in nodes of the SBN after only 5 min of a social interaction. An aggressive conspecific stimulus caused a modulation of DHEA levels in specific brain areas differently in both seasons (Heimovics et al., 2016). Together, these data suggest that song sparrows modulate local levels of steroids in the brain in response to social interactions and this response is season-specific.
1.8 Steroid measurement in brain

In order to understand the effects of aggressive encounters on neurosteroid production, we need to be able to accurately measure neurosteroid levels. The Palkovits punch technique (Palkovits, 1973), has been used in several avian species (Schumacher and Balthazart, 1987; Charlier et al., 2011; Heimovics et al., 2016). Thick (e.g. 300 μm) sections of brain tissue are cut on a cryostat, and cannulae with inner diameters ranging from 250 μm to 2mm are used to microdissect tissue from multiple brain regions. The quantities of tissue collected in these “punches” are very small. For example, one punch that is 300 μm thick and 1mm in diameter weighs 0.245 mg (Taves et al., 2011). Multiple punches from the same region can be pooled to obtain sufficient tissue to measure steroids present at low concentrations. Because the brain contains many lipids that can interfere with steroid assays, liquid-liquid or solid phase extraction should be used to separate steroids from interfering substances (Appelblad and Irgum, 2002; Taves et al., 2011).

Sex steroids are potent signaling molecules that can exert strong effects at very low concentrations, and therefore measuring the very low levels that are found in biological samples constitutes a major analytical challenge. Detecting rapid changes in neurosteroids in punches, particularly with high spatial resolution, might require greater sensitivity than is possible with immunoassays. Also, although immunoassays have been widely used to quantify steroids in brain tissue, one of the problems with these assays is the specificity. Other steroids in the sample can cross-react to the antibodies and therefore concentrations can be overestimated when using immunoassays (Grebe and Singh, 2011). Moreover, because levels of multiple steroids can change concomitantly within the same brain region, an ideal assay would allow measurement of other active metabolites of DHEA, in addition to T and 17β-E2, such as androstenedione,
androstenediol, estrone, and 17α-estradiol. To address this need, we are currently developing novel protocols for LC-MS/MS (Tobiansky et al., 2018), an ultrasensitive method that allows the measurement of multiple analytes with great specificity (Vogeser and Parhofer, 2007). Liquid chromatography separates the components of the sample whereas the mass spectrometry provides structural identity of the individual components providing high analytical specificity and sensitivity (Vogeser and Parhofer, 2007).

1.9 Estrogen derivatization

LC-MS/MS sensitivity for 17β-E₂ measurement is limited due to the low ionization efficiency of estrogens. Nevertheless, this can be improved by chemical derivatization which increases ionization efficiency of analytes by adding a highly ionizable moiety to the analyte (Faqehi et al., 2016; Heimovics et al., 2018).

The most widely used reagent for 17β-E₂ derivatization is dansyl chloride (DSCL), a method that has been validated in different mammal species such as rats, monkeys, and humans and in different sample types such as serum and brain. DSCL interacts with the hydroxyl group on the phenolic ring of 17β-E₂ which enables selective derivatization of estrogens. Since other steroids lack a phenolic group, they are not reactive with DSCL (Anari et al., 2002). This simplifies the analysis and it also allows the quantification of other steroids in the same sample without interference. However, a disadvantage is that DSCL estrogen derivatization quantification is not as specific since the product ion is generated by the dansyl moiety and therefore is not specific for the analyte by mass.

In contrast, the derivatization reagent 1,2-dimethylimidazole-5-sulfonyl chloride (DMIS) has an analyte-specific fragmentation that enables monitoring of confirmatory mass transitions
with high sensitivity (Keski-Rahkonen et al., 2015). Other derivatization reagent recently used to derivatize estrogens is 1-methylimidazole-2-sulfonyl chloride (ISCL) (Li and Franke, 2015), which increases sensitivity in serum samples respect to DSCL, and it might facilitate the profiling of estrogens in brain tissue, but it has not been tested yet. These methodological advances will enable us to test whether an aggressive interaction rapidly modulates levels of 17β-E₂ and other estrogens in a region-specific manner in songbirds, and they will also be useful for studies of estrogens in rodents and humans.

1.10 Objectives

In this dissertation, I test the hypothesis that the brain regulates local steroid levels throughout the year and that aggressive interactions rapidly stimulate local neurosteroid synthesis in the brain only during the nonbreeding season. I present three studies examining the balance of peripheral and local production of steroids in the song sparrow brain.

In Chapter 2, I developed a novel LC-MS/MS method to measure a panel of 10 steroids in the blood and discrete brain regions of song sparrows. I used this method to carry out a seasonal study of circulating and local levels of steroid hormones with a focus on androgens and their precursors in wild song sparrows. The liquid-liquid extraction protocol is fast, simple and effective and the steroid assay is highly accurate, precise, specific and sensitive. We found a marked seasonality in systemic and brain steroid levels. As expected, androgen and estrogen concentrations were higher in the breeding season than in the non-breeding season. Interestingly, in breeding males, testosterone levels were higher in the circulation than brain, whereas 5α-dihydrotestosterone levels were up to 20-fold higher in specific brain regions than in blood. Moreover, in the breeding season, estrogens were detectable in brain but not in blood.
Progesterone showed seasonal differences in several brain regions despite the lack of seasonal changes in blood. Corticosterone levels in the blood were higher in the breeding season than in the non-breeding season, but corticosterone levels showed few seasonal differences in the brain. Our data show that brain steroid levels can differ greatly from circulating steroid levels in the blood. Further, brain steroid levels show region-specific seasonal patterns that are not a simple reflection of circulating steroid levels.

In Chapter 3, I developed a method to measure several estrogens in the blood and discrete brain regions of song sparrows. We combined estrogen derivatization with LC-MS/MS to achieve high sensitivity and used this method to carry out a seasonal study of circulating and local levels of four estrogens simultaneously in wild song sparrows. The straightforward protocol improved sensitivity by 10-fold for some analytes. There is substantial regional variation in neuroestrogen levels in brain areas that regulate social behavior in male song sparrows. For example, the auditory area NCM, which has high aromatase levels, has the highest estrone and 17β-\(E_2\) levels. In contrast, estrogen levels in blood are very low. Estrogen levels in both brain and circulation are lower in the non-breeding season than in the breeding season. This technique will be useful for estrogen measurement in songbirds and potentially other animal models.

In Chapter 4, I explored the acute effects of an aggressive interaction on circulating and brain steroid levels across seasons. We applied the LC-MS/MS method developed in chapter 2 to measure steroids in the blood and discrete brain regions of song sparrows and carried out a seasonal study with a focus on androgens and their precursors in wild song sparrows. Overall, our data showed a precise local steroid regulation in the brain of both breeding and non-breeding wild song sparrows. Particularly, in the non-breeding season, an aggressive interaction induced rapid increases in T concentrations in the brain, while T levels were not detectable in the general
circulation. Further, the effects of the brief social interaction on progesterone levels in non-breeding animals were different in the brain than in the circulation. In contrast, during the breeding season, progesterone, T, and 5α-DHT levels were not acutely modulated by the aggressive interaction. These data suggest that local levels of steroids are rapidly modulated by aggressive interactions during the non-breeding season in a region-specific manner.
1.11 Tables and figures

Figure 1.1: The social decision-making network

Figure from O’Connell and Hofmann 2011. Brain regions in the social behavior network (left) and mesolimbic reward system (right).
Figure 1.2: Steroid synthetic pathway

Steroid names are bold and steroidogenic enzymes in italics. By: M. Salehzadeh
Chapter 2: Profiling of systemic and brain steroids in male songbirds: Seasonal changes in neurosteroids

2.1 Introduction

Steroids are important modulators of neurophysiology and behavior that are secreted into the circulation by organs such as the gonads and adrenal glands. In addition, the brain itself is capable of producing steroids (Baulieu, 1991). Steroids synthesized locally within the brain are known as neurosteroids (Jalabert et al., 2018), and they can be produced either by the local conversion of circulating prohormones or de novo from cholesterol (Figure 2.1). Neurosteroids are present in all classes of vertebrates, including fish (Diotel et al., 2011), amphibians (Vaudry et al., 2011), reptiles (Peek and Cohen, 2018), birds (Tsutsui, 2011; Schlinger, 2015), and mammals (Hojo et al., 2008; Hojo and Kawato, 2018).

As steroids can be synthesized locally in the brain, steroid levels in the blood often do not reflect steroid levels in specific brain regions. Thus, it is critical to accurately measure steroid levels in discrete brain regions. This can be achieved by combining brain microdissection with a sensitive steroid assay (Heimovics et al., 2018). While immunoassays are widely used to measure steroids, they have potential limitations, including lack of sensitivity to measure steroids in small amounts of brain tissue (1-2 mg), measurement of only one analyte, and importantly, antibody cross-reactivity with similar analytes (Grebe and Singh, 2011). Antibodies typically cross-react with structurally similar steroids, and thus steroid concentrations are overestimated by immunoassays, especially at low analyte levels (Wudy et al., 2018; Hamden et al., 2019).
Steroids can be measured by liquid chromatography-tandem mass spectrometry (LC-MS/MS), an ultrasensitive technique that allows the simultaneous measurement of multiple analytes in the same sample. Very briefly, the LC separates the steroids, and the tandem mass spectrometer monitors transitions of specified precursor ions to specified product ions at particular retention times (Vogeser and Parhofer, 2007). In this way, LC-MS/MS measures multiple steroids in a single run with very high analytical specificity.

Song sparrows, *Melospiza melodia*, are an excellent animal model for using LC-MS/MS to examine steroid levels in the blood and specific brain regions. Songbirds show high rates of steroid synthesis in the brain (Remage-Healey *et al.*, 2017), facilitating steroid measurement. Moreover, some song sparrows aggressively maintain territories year-round (except during molt). During the breeding season, males are highly territorial and aggressive, and this behavior is supported by testosterone (T) from the testes. Interestingly, non-breeding males also defend territories aggressively, although the testes are regressed and androgen levels in the circulation are very low (Wingfield and Hahn, 1994). Furthermore, castration does not reduce aggression in the non-breeding season (Wingfield, 1994).

Nonetheless, aromatase, which converts androgens into estrogens, in song sparrows is highly expressed in specific brain regions during the non-breeding season (Soma *et al.*, 2003; Wacker *et al.*, 2010), and inhibition of aromatase reduces non-breeding aggression (Soma, Sullivan and Wingfield, 1999; Soma *et al.*, 2000; Soma, Tramontin and Wingfield, 2000). These data suggest that neurosteroids such as T and 17β-estradiol (17β-E2) regulate non-breeding aggression in this species. Neurosteroids could be locally synthesized from circulating dehydroepiandrosterone (DHEA), progesterone and/or pregnenolone (Soma and Wingfield, 2001; Vaudry *et al.*, 2011; Fokidis *et al.*, 2019), or neurosteroids could be locally synthesized
from cholesterol in the brain (Fokidis et al., 2019). Songbirds, including song sparrows, express several steroidogenic enzymes in the brain (Soma et al., 2003; Pradhan et al., 2010; Wacker et al., 2010).

Brain steroidogenic enzymes and neurosteroid levels show region-specific seasonal changes in song sparrows. In the diencephalon and telencephalon 3β-hydroxysteroid dehydrogenase/Δ5–Δ4 isomerase (3β-HSD) activity is higher during the non-breeding season than the breeding season and molt. Further, an aggressive interaction rapidly increases 3β-HSD activity in the telencephalon during the non-breeding season (Pradhan et al., 2010). Similarly, brain aromatase shows region-specific seasonal changes. Aromatase activity in the ventromedial telencephalon is reduced during molt relative to the breeding and non-breeding seasons (Soma et al., 2003). In captive breeding and non-breeding males, DHEA, T, and 17β-E2 levels (as measured by radioimmunoassays) are higher in microdissected brain regions than in the circulation. In both seasons, a very short (5 min) aggressive interaction modulates levels of DHEA in specific brain areas but not in the blood (Heimovics et al., 2016). However, captivity causes a decrease in sex steroids due to the lack of full social stimuli and therefore wild animals offer a better opportunity to study the natural seasonal changes in steroid levels. Other bioactive steroids such as 5α-dihydrotestosterone (5α-DHT) and estrone have not been assessed in the songbird brain.

Here, we developed and validated a LC-MS/MS assay for the measurement of 10 steroids in whole blood (hereafter “blood”), plasma, and microdissected brain tissue (1-2 mg) of male song sparrows. We then examined seasonal changes of steroids in blood, plasma, and 10 brain regions that regulate social behavior, in free-living territorial males. Our panel included 10 steroids: pregnenolone, progesterone, corticosterone, DHEA, androstenedione, T, 5α-DHT,
estrone, 17β-E₂, and 17α-estradiol (Figure 2.1). This panel provides a comprehensive picture of
the steroidal environment in the circulation and brain of wild animals, in order to examine
possible neural synthesis of steroids in wild songbirds.

2.2 Materials and methods

2.2.1 Field procedures

Free-living adult male song sparrows were captured in the non-breeding season (January
24th to February 2nd, 2017) and breeding season (May 8th to May 19th, 2017) (n = 10 per
season). Subjects were captured near Vancouver, BC using a mist net and conspecific song
playback for a maximum of 5 min (breeding: 1.2 ± 0.6 min, non-breeding: 1.9 ± 0.5 min; p =
0.40). Immediately after a subject was caught in the net, the subject was rapidly and deeply
anesthetized with isoflurane and then rapidly decapitated (breeding: 2.6 ± 0.2 min, non-breeding:
1.4 ± 0.3 min; p = 0.21). It was a maximum of 3 min between catching the subject to euthanasia,
to avoid effects of handling on steroid levels. The brain was immediately dissected from the skull
and snap frozen on powdered dry ice. Trunk blood was collected in heparinized microhematocrit
tubes (Fisher Scientific) that were kept on ice packs until returned to the laboratory (within 5h).
Once in the laboratory, blood was divided into 2 aliquots; one half of the blood sample was
frozen and the other half was centrifuged and plasma was collected. All samples were stored at –
80°C until steroids were extracted.

All procedures were in compliance with the Canadian Council on Animal Care and
protocols were approved by the UBC Animal Care Committee.
2.2.2 Brain microdissection

The Palkovits punch technique was used to microdissect brain tissue from 10 brain areas, including nodes in the social decision-making network (SDMN) (O’Connell and Hofmann, 2011): preoptic area (POA), anterior hypothalamus (AH), lateral septum (LS), bed nucleus of the stria terminalis (BnST), ventromedial hypothalamus (VMH), ventral tegmental area (VTA), central gray (CG), and nucleus taeniae of the amygdala (TnA). We also collected the caudomedial nidopallium (NCM) which expresses high aromatase and is involved in song perception (Bolhuis and Gahr, 2006) and cerebellum (Cb) which expresses little aromatase (Fusani et al., 2000; Charlier et al., 2011). Punch location for each brain area is shown in Figure 2.2. Brains were sectioned in the coronal plane at 300 μm on a cryostat at –14°C using a plane of sectioning that closely matched a zebra finch brain atlas (Nixdorf-Bergweiler and Bischof, 2007) as before (Heimovics et al., 2016). Sections were mounted onto Superfrost Plus microscope slides (Thermo Fisher Scientific Inc.) and kept cold on dry ice during punching (Tobiansky et al., 2018). Specific sections containing the brain regions of interest were microdissected using an Integra Miltex stainless-steel biopsy punch tool (1 mm diameter, tissue wet weight 0.245 mg per punch (Taves et al., 2011), Fisher Scientific). The same punch size was used for all brain areas. Depending on the size of the brain area, 4 or 6 punches were collected (total from both sides) and tissue amount was 0.98 or 1.47mg, respectively.

Brain microdissection was performed as before (Heimovics et al., 2016). Specifically, four punches (2/side) containing the POA were collected from two serial sections immediately caudal to the last section containing the tractus septopalliomesencephalicus (TrSM). Four punches (2/side) containing the AH were collected from two serial sections immediately caudal to the POA sections and ventral to the anterior commissure (CoA). POA and AH punches were
immediately lateral to the midline. Four punches (2/side) containing the LS and BnST were collected dorsal to the CoA, the LS medial to the lateral ventricles and the BnST at the tip of each lateral ventricle. Four punches (2/side) containing the VMH were collected ventral to the AH. Four punches (2/side) containing the VTA were collected from two serial sections ventrolateral to NIII. Four punches (2/side) containing the CG were collected from two serial sections ventral to the posterior commissure. Six punches (2/side) containing the NCM were collected from three serial sections, one section rostral to TnA and two sections with TnA. NCM punches did not include the song control nucleus HVC. Four punches (2/side) containing the TnA were collected from two serial sections caudal to the disappearance of the CoA and tractus occipito-mesencephalicus path from the ventromedial telencephalon. Six punches (centered in the midline) containing the Cb were collected from 3 sections from its first appearance. Punches were expelled into 2-mL polypropylene vials (Sarstedt AG & Co.) kept on dry ice. Vials were stored at −80°C until processing.

Circulating levels of steroids were measured in both blood (5 µL per sample) and plasma (10 µL per sample). We used blood to assess circulating steroid levels and to compare with brain steroid levels, because the use of plasma overestimates steroid concentrations in the circulation (Taves et al., 2010, 2011, 2015). We used plasma to match previous reports in song sparrows.

### 2.2.3 Steroid extraction

Steroids were extracted from blood, plasma, and brain tissue using liquid-liquid extraction, as before (Mohr et al., 2019; Tobiansky et al., 2020). Briefly, five zirconium ceramic oxide beads (1.4-mm diameter, Fisher Scientific) were added to each vial. Then 50 µL of the deuterated internal standards (pregnenolone-d4, progesterone-d9, corticosterone-d8, DHEA-d6,
testosterone-d5, 17β-estradiol-d4; C/D/N Isotopes Inc., Pointe-Claire, Canada) in 50% HPLC-grade methanol were added to each sample (and calibration curves) to track recovery and matrix interference for each sample. After adding 1 mL HPLC-grade acetonitrile (ACN) to each vial, samples were homogenized using a bead mill homogenizer at 4 m/s for 30 s (Omni International Inc., Kennesaw, GA). Then samples were centrifuged at 16,100g for 5 min, and 1 mL of supernatant was transferred to a borosilicate glass culture tube pre-cleaned with HPLC-grade methanol (VWR International). Then 0.5 mL of HPLC-grade hexane was added, and samples were vortexed and centrifuged at 3200g for 2 min. The hexane was removed and discarded, and the ACN was dried in a vacuum centrifuge at 60°C for 45 min (ThermoElectron SPD111V). Samples were reconstituted with 55 μL of 25% HPLC-grade methanol in MilliQ water, transferred to 0.6 mL polypropylene microcentrifuge tubes (Fisher Scientific), and centrifuged at 16,100g for 2 min. Then 50 μL of supernatant were transferred to a LC vial insert (Agilent, Santa Clara, CA) and stored overnight at -20°C until injection.

Samples were processed along with blanks and calibration curves. Calibration curves were made from certified reference standards (Cerilliant Co., Round Rock, TX) prepared in 50% HPLC-grade methanol. The calibration curve range was 0.05 to 1000 pg/tube for progesterone and T; 0.1 to 1000 pg/tube for corticosterone, 17α-estradiol, and estrone; 0.2 to 1000 pg/tube for androstenedione, 5α-DHT, and 17β-E2; 4 to 10,000 pg/tube for DHEA; and 12.5 to 1000 pg/tube for pregnenolone (Table 2.1).

2.2.4 **Steroid analysis by LC-MS/MS**

Steroids were quantified using a Sciex QTRAP 6500 UHPLC-MS/MS system as previously described (Tobiansky *et al.*, 2018). Samples were transferred into a refrigerated
autoinjector (15°C). Then, 45 μL of resuspended sample were injected into a Nexera X2 UHPLC system (Shimadzu Corp., Kyoto, Japan), passed through a KrudKatcher ULTRA HPLC In-Line Filter (Phenomenex, Torrance, CA) followed by a Poroshell 120 HPH C18 guard column (2.1 mm) and separated on a Poroshell 120 HPH C18 column (2.1 x 50 mm; 2.7 μm; at 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. During loading, MPB was at 10% for 0.5 min, from 0.6 to 4 min the gradient profile was at 42% MPB, which was ramped to 60% MPB until 9.4 min. From 9.4 to 9.5 min the gradient was 60-70% MPB, which was ramped to 98% MPB until 11.9 min and finally a column wash from 11.9 to 13.4 min at 98% MPB. The MPB was then returned to starting conditions of 10% MPB for 1 min. Total run time was 14.9 min. The needle was rinsed externally before and after each sample injection with 100% isopropanol.

We used 2 multiple reaction monitoring (MRM) transitions for each steroid and 1 MRM transition for each deuterated internal standard (Table 2.2). Steroid concentrations were acquired on a Sciex 6500 Qtrap triple quadrupole tandem mass spectrometer (Sciex LLC, Framingham, MA) in positive electrospray ionization mode for all steroids except estrone, 17β-E2, and 17α-estradiol, which were acquired in negative electrospray ionization mode (Figure 2.3).

All water blanks were below the lowest standard on the calibration curves. Matrix effects were assessed by creating blood, plasma, and brain pools, which were used to make serial dilutions and evaluate linearity and parallelism to calibration curves (Table 2.3). Recovery was assessed for blood, plasma, and brain pools by comparing unspiked samples with samples spiked with a known amount of steroid. Accuracy was assessed by measuring a quality control with a known concentration in neat solution (Table 2.4). Precision was evaluated by comparing
replicates of a quality control within runs (intra-assay variation) and across runs (inter-assay variation) (Table 2.4). Furthermore, we measured the retention time of 5β-DHT, an isomer of 5α-DHT, and with the chromatography conditions used here, the retention times were quite different (over 30 sec). Thus, our measure of 5α-DHT is not affected by 5β-DHT levels.

2.2.5 Statistical analysis

A value was considered non-detectable if it was below the lowest standard on the calibration curve. When detectable samples in a group were ≥ 20%, missing values were estimated via quantile regression imputation of left-censored missing data using MetImp web tool (Wei, Wang, Jia, et al., 2018; Wei, Wang, Su, et al., 2018; Tobiansky et al., 2020). When detectable samples in a group were < 20%, non-detectable values were set to 0. To make a comparison between steroid levels in brain and blood, we assumed that 1 mL of blood weighs 1 g (Taves et al., 2011; Tobiansky et al., 2020).

Statistics were conducted using GraphPad Prism version 6.00 (GraphPad Software, La Jolla, CA). When necessary, data were log transformed prior to analysis. Seasonal variations in blood and plasma steroid levels were analyzed by t-tests, and non-parametric Mann-Whitney U tests were used when imputations were not possible (i.e., detectable samples were < 20%). Pearson correlation analyses were used to examine the relationships between circulating androgens, as well as between blood and plasma levels of the same steroid. Regional differences in local androgens and estrogens levels were analyzed by repeated measures one-way analysis of variance (ANOVA), as different brain areas are collected from the same individual. Analyses were followed by Tukey multiple comparison tests and corrected p values are shown. For corticosterone and progesterone, a repeated measures two-way ANOVA with one between-
subjects factor (season) and one within-subjects factor (sample type) was used to examine seasonal changes of these steroids in blood and brain regions. Analyses were followed by Sidak multiple comparison tests and corrected p values are shown. Significance criterion was set at p ≤ 0.05. Graphs show the mean ± standard error of the mean (SEM) and are presented using the non-transformed data.

2.3 Results

2.3.1 Androgen levels in blood and plasma

As expected, in blood, androgen levels in male song sparrows were higher in the breeding season than the non-breeding season (Figure 2.4 A-C). Androstenedione and 5α-DHT were non-detectable in all non-breeding subjects and detectable in all breeding subjects, and these seasonal differences were statistically significant (p < 0.0001 in both cases; Figure 2.4 A, C). Blood T levels were also significantly higher in breeding subjects than non-breeding subjects (p < 0.0001; Figure 2.4B).

In breeding subjects, blood androstenedione and T levels were significantly positively correlated ($r^2 = 0.536$, $p = 0.025$; Figure 2.4D) and blood T and 5α-DHT levels were also positively correlated ($r^2 = 0.855$, $p = 0.0001$; Figure 2.4E).

In breeding subjects, plasma androgen levels were positively correlated with blood androgen levels (all p values ≤ 0.001) but approximately 2-fold higher, as we have seen before (Taves et al., 2010) (Table 2.5).
2.3.2 Androgen levels in microdissected brain regions

In breeding subjects, androstenedione, T, and 5α-DHT were detectable in microdissected brain regions. However, in non-breeding subjects, androstenedione, T, and 5α-DHT were non-detectable in all brain samples. Thus, we examined regional variation in brain androgen levels in breeding subjects only.

One-way repeated measures ANOVAs was conducted to compare androgen levels across the different brain regions in breeding subjects. For androstenedione, there was a significant effect of brain region (F_{8,72} = 17.94, p = 0.0001; Figure 2.5A). Post-hoc comparisons revealed that NCM had significantly higher androstenedione levels than POA, AH, BnST, TnA, and Cb (all p values ≤ 0.05). Androstenedione levels did not differ among NCM, VTA, VMH, and CG, nor among POA, AH, BnST, CG and TnA or VMH, VTA, and TnA. Androstenedione levels were non-detectable in the LS.

T levels in breeding subjects also showed a significant effect of brain region (F_{9,81} = 26.18, p < 0.0001; Figure 2.5B). Post-hoc comparisons revealed that VTA had significantly higher T levels than POA, AH, LS, BnST, VMH, and TnA (all p values < 0.01). T levels were not different among VTA, CG, Cb, and NCM, nor among LS, AH, VMH, and TnA. T levels were the lowest in the BnST.

5α-DHT levels in breeding subjects showed a significant effect of brain region (F_{9,81} = 20.38, p < 0.0001; Figure 2.5C). 5α-DHT levels were high and not different among POA, AH, LS, VMH, VTA, CG, and TnA. The next highest levels of 5α-DHT were in NCM and Cb. Levels of 5α-DHT were lowest in the BnST.

Androgen levels in the brain and blood were compared in breeding subjects only (Table 2.6). Androstenedione levels were higher in the brain than in the blood (brain:blood ratio, range
2.8 - 6.1), except in the LS and Cb. In contrast, T levels were lower in all brain regions than in the blood (brain: blood ratio, range 0.3 - 0.7). As expected, 5α-DHT levels were higher in the brain than in the blood (brain: blood ratio, range 6.1 - 19.9).

2.3.3 DHEA levels in blood, plasma, and brain

Unexpectedly, DHEA was not detected in blood, plasma, or brain samples. DHEA levels were non-detectable in blood, plasma, or brain tissue (see Table 2.1 for lower limit of quantification (LLOQ)).

In contrast, previous studies using immunoassays detected DHEA immunoreactivity in plasma (approximately 0.8 – 1.5 ng/mL) and brain samples (approximately 2 – 6 ng/g) (Soma and Wingfield, 2001; Soma et al., 2002; Goodson, Evans and Soma, 2005; Newman, Pradhan and Soma, 2008; Scotti et al., 2009; Newman et al., 2010, 2013; Newman and Soma, 2011; Maddison et al., 2012; Heimovics et al., 2016).

This difference could be the result of differences in assay sensitivities. The sensitivity of the present LC-MS/MS assay for DHEA (4 pg/tube) was slightly worse than the sensitivities of the previous radioimmunoassays for DHEA (2 pg/tube). Thus, to improve sensitivity for DHEA, we used derivatization with hydroxylamine and quantified DHEA in plasma by LC-MS/MS, as we did before (Fokidis et al., 2019). After derivatization with hydroxylamine, the DHEA LLOQ was improved (2.5 pg). Following derivatization, DHEA was indeed detectable in plasma (50 µL), but plasma DHEA levels were generally very low, with a significant seasonal difference (breeding: 0.15 ± 0.05 ng/mL, non-breeding: 0.03 ± 0.008 ng/mL; p = 0.007).

These plasma DHEA levels are at least 10-fold lower than in previous reports that used radioimmunoassays. This difference could be the result of differences in assay specificity,
especially cross-reactivities of the anti-DHEA antibodies with similar steroids. Therefore, we used LC-MS/MS to examine the presence of multiple DHEA metabolites in plasma. Steroids were extracted from 50µL of plasma using liquid-liquid extraction followed by solid-phase extraction (Tobiansky et al., 2018). 7β-OH-DHEA (LLOQ 0.4 ng/mL), androstenediol (LLOQ 1 ng/mL), 7α-OH-DHEA and 16α-OH-DHEA (LLOQ 2.5 ng/mL), were all non-detectable in plasma from breeding and non-breeding subjects.

2.3.4 Estrogen levels in blood and plasma

Not surprisingly, in blood and plasma, estrone, 17β-E2 and 17α-estradiol were non-detectable in all breeding and non-breeding males.

2.3.5 Estrogen levels in microdissected brain regions

In breeding subjects, estrone and 17β-E2 were detectable in some microdissected brain regions. However, in non-breeding subjects, estrone and 17β-E2 were non-detectable in all brain samples. Thus, we examined regional variation in brain estrone and 17β-E2 levels in breeding subjects only. 17α-estradiol was non-detectable in all brain regions in both seasons.

One-way repeated measures ANOVAs were conducted to compare estrogen levels across the different brain regions in breeding subjects. For estrone, there was a significant effect of brain region (F₆,₅₄ = 75.58, p < 0.0001; Figure 2.6A). Post-hoc comparisons revealed that estrone concentrations were higher in NCM than other brain regions, as expected (all p values < 0.001). No differences in estrone levels were found among BnST, VMH, and TnA, nor among POA, AH, and TnA. Estrone levels were lower in the CG (p < 0.0001 in all cases) and non-detectable in the LS, VTA, and Cb.
17β-E2 levels in breeding subjects showed a significant effect of brain region (F4,36 = 14.55, p < 0.0001; Figure 2.6B). Post-hoc comparisons revealed that 17β-E2 concentrations were higher in NCM than other brain regions, as expected (all p values ≤ 0.01). 17β-E2 levels in the POA, AH, and VMH were not different from each other nor between AH and TnA. 17β-E2 was non-detectable in LS, BnST, VTA, CG, and Cb. Overall, estrone and 17β-E2 levels showed similar regional differences, with highest levels in NCM, which expresses high levels of aromatase.

2.3.6 Progesterone, corticosterone, and pregnenolone levels in blood, plasma, and brain

Progesterone was detectable in most blood, plasma and brain samples. For progesterone, there was a significant main effect of sample type (blood or brain region) (F10,180 = 12.29, p < 0.0001; Figure 2.7A). There was also a significant main effect of season (F1,18 = 6.09, p = 0.024) and a significant sample type X season interaction (F10,180 = 3.11, p = 0.001). Interestingly, post-hoc comparisons revealed that progesterone levels did not show seasonal differences in the blood, but did show seasonal differences in several brain regions. Progesterone levels were higher in the non-breeding season than the breeding season in POA, AH, VMH, TnA, NCM, and Cb (all p values < 0.01). LS, BnST, VTA, and CG did not show seasonal changes in progesterone levels. Plasma progesterone levels were positively correlated with blood progesterone levels (r² = 0.938, p < 0.0001) but approximately 2-fold higher (Table 2.5).

Corticosterone was detectable in all blood, plasma, and brain samples. There was a significant main effect of sample type (blood or brain region) (F10,180 = 105.4, p < 0.0001; Figure 2.7B). There was also a significant main effect of season (F1,18 = 6.713, p = 0.018) and a significant sample type X season interaction (F10,180 = 7.383, p < 0.0001). Post-hoc comparisons
revealed that breeding subjects had significantly higher blood corticosterone levels than non-breating subjects (p < 0.0001). In contrast, corticosterone levels in POA, AH, LS, BnST, VMH, TnA, NCM, and Cb did not show seasonal changes. Corticosterone levels were higher in the breeding season than the non-breeding season in the VTA (p = 0.0345) and CG (p = 0.0317), although to a lesser extent than in the blood (Figure 2.7B). Plasma corticosterone levels were positively correlated with blood corticosterone levels ($r^2 = 0.974$, p < 0.0001) but approximately 2-fold higher, as we have seen before (Taves et al., 2010) (Table 2.5).

Pregnenolone levels were non-detectable in blood, plasma, or brain tissue (see Table 2.1 for LLOQ).

### 2.4 Discussion

Here, we measured a panel of 10 steroids in blood, plasma, and 10 microdissected brain areas, yielding 120 data points per subject and a comprehensive picture of the diverse steroidal environments in the circulation and specific brain regions. The sample preparation method that we developed is straightforward, rapid, economical, and effective at removing matrix interference, and it is thus conducive to high-throughput studies. The LC-MS/MS assay is highly accurate, precise, sensitive, and specific and does not involve derivatization, which makes it simpler and more robust. This is the first study to (1) describe natural seasonal changes in brain steroid levels with great spatial resolution in any species and (2) measure a large panel of sex steroids such as 5α-DHT and 17β-E2 as well as their precursors in songbirds. Here, we observed several novel findings with regard to the SDMN. First, in breeding males, androstenedione and 5α-DHT concentrations can be higher in the brain than in the blood, as much as 20-fold higher. Second, estrone and 17β-E2 are detectable in brain areas that regulate aggressive behavior, but
not in the blood, of breeding males. Third, brain progesterone levels are often higher in the non-breeding than breeding season, in contrast to the blood, where no seasonal difference is present. Fourth, corticosterone levels are lower in the brain than in the blood, and a seasonal change in corticosterone is robust in the blood but less so in the brain. Finally, all steroids measured showed different concentrations across the brain areas studied. Taken together, these data provide fundamental insights into local steroid regulation in the SDMN.

2.4.1 Development of steroid extraction and quantification protocols

We were able to accurately and precisely measure a panel of 10 steroids in 5 µL of blood, 10 µL of plasma, and ~1 to 1.5 mg of brain tissue. Previous studies have generally used gross dissection to measure steroids in the brain, which lacks spatial resolution. Further, complex sample preparation was required due to the large amount of tissue used. In contrast, here we used 1-mm Palkovits punch for microdissection and collected 4 to 6 punches per region. We estimate that each punch contains ~92,000 cells (~64,000 neuronal and 28,000 non-neuronal cells) (Olkowicz et al., 2016) and that each sample contains hundreds of thousands of cells. In the future, even greater spatial specificity may be possible, via laser capture microdissection or mass spectrometry imaging.

The liquid-liquid extraction protocol is straightforward, fast, and more economical than solid-phase extraction protocols, yields low background values (increasing sensitivity), produces high and consistent analyte recoveries, and effectively removes matrix interference. First, serial dilutions of blood, plasma, and brain show linearity and parallelism with the calibration curves. Second, deuterated internal standards do not show ion suppression or enhancement in the
different matrices. Third, recovery experiments (comparing unspiked and spiked samples) confirm the lack of matrix effects.

LC-MS/MS offers several major benefits over immunoassays. First, LC-MS/MS allows measurement of multiple steroids in the same sample during a single run, including steroids that are not commonly measured, such as androstenedione, estrone, and 17α-estradiol. Thus, one can gain a much broader understanding of the systemic and local steroidal environments, relative to a narrow focus on a few steroids, such as T and corticosterone. Second, sensitivity is similar or better with LC-MS/MS. Here, we were able to measure as little as 0.05 to 0.2 pg of most of the steroids, using electrospray ionization and a very sensitive detector. Future work can employ derivatization or microflow LC to improve sensitivity even further. Third, LC-MS/MS is extremely specific. With immunoassays, antibodies can cross-react with closely-related steroids and thus overestimate analyte concentrations, especially at low analyte concentrations (Grebe and Singh, 2011; Wudy et al., 2018). This is a major and underappreciated problem. With LC-MS/MS, multiple criteria are used to unequivocally quantify analytes. Analyte precursor ions are selected by mass and then fragmented. Transitions to two product ions are monitored (quantifier ion and qualifier ion), and the ratio between the quantifier and qualifier ions is calculated and compared to that of the standards. Further, the retention time is specific to the analyte and allows better selectivity. Overall, our method allows for the measurement of multiple steroids with high analytical specificity and sensitivity and will be broadly useful, opening the door to many new possibilities in neuroendocrinology.
2.4.2 Seasonality of circulating steroids

All steroid concentrations were approximately twice as high in plasma as in blood, as before (Taves et al., 2010, 2011). Steroid levels in blood are a better estimation of levels in circulation, and the use of plasma samples overestimates circulating steroid concentrations. Therefore, blood levels are better to compare with brain levels, as a potential indication of local steroid synthesis (Taves et al., 2010, 2011, 2015).

As expected, circulating androgen levels showed a marked seasonal pattern. Breeding subjects had higher levels of circulating androstenedione, T, and 5α-DHT than non-breeding subjects, similar to previous studies (Soma and Wingfield, 1999; Heimovics, Fokidis and Soma, 2012; Fokidis et al., 2019). This is a very common pattern in male vertebrates (Adkins-Regan, 2005). In most vertebrates, these seasonal changes in circulating androgens parallel seasonal changes in territorial aggression (Jalabert et al., 2018). However, male song sparrows are highly aggressive during both the breeding and non-breeding seasons, despite dramatic reductions in circulating androgens during the non-breeding season (Wingfield and Hahn, 1994). Plasma androstenedione, T, and 5α-DHT levels seen here are similar to previous reports in this species (Wingfield and Hahn, 1994; Soma et al., 2003; Pradhan et al., 2010; Fokidis et al., 2019). We also examined 17α-testosterone (or epitestosterone) in song sparrow plasma and observed very low concentrations (<0.1 ng/mL), that were slightly higher in the breeding season than the non-breeding season (C. Jalabert, unpublished results).

Circulating levels of progesterone did not show seasonal changes, and plasma levels are comparable to those reported before in this species with LC-MS/MS (Fokidis et al., 2019). Similarly, male white-crowned sparrows show no difference in plasma progesterone when housed under long days or short days (McCreery and Farner, 1979). In contrast, the house
sparrow shows seasonal fluctuations in circulating progesterone, with higher levels in the breeding season than the non-breeding season (Bharucha and Padate, 2009). Circulating progesterone has been linked to aggression in birds. In male ovenbirds, an aggressive interaction increases plasma progesterone during the breeding season (Adreani, Goymann and Mentesana, 2018). In contrast, an aggressive interaction decreases plasma progesterone in breeding male white-crowned sparrows (Charlier et al., 2009). Similarly, in the sex-role reversed black coucal, circulating progesterone in females decreases after an aggressive interaction and progesterone administration reduces aggression (Goymann et al., 2008). Pregnenolone was non-detectable in blood or plasma consistent with previous reports with LC-MS/MS as expected values were below our LLOQ (Fokidis et al., 2019).

Circulating corticosterone levels show large seasonal changes. In many avian species, circulating corticosterone levels are highest in the breeding season, as seen here (Romero, 2002). The present data are consistent with previous studies on song sparrows, and may be related to the need for energy mobilization during the breeding season, for reproductive and parental behavior (Newman, Pradhan and Soma, 2008; Newman and Soma, 2009). Further, the baseline plasma corticosterone levels seen here are similar to our previous reports (Newman and Soma, 2009; Newman et al., 2010).

In the present study, we did not detect estrone, 17α-estradiol, or 17β-E2 in the blood and plasma of male song sparrows. In previous studies of male song sparrows that used radioimmunoassays, plasma 17β-E2 was generally non-detectable, except for a small increase at the beginning of the breeding season (Soma and Wingfield, 1999). Very few studies have measured estrone in songbirds (Schlinger and Arnold, 1992), and no other study has measured 17α-estradiol in songbirds.
Without derivatization, DHEA was non-detectable in blood and plasma. In a follow-up study, we used derivatization to increase assay sensitivity. With derivatization, plasma DHEA concentrations are detectable but low (0.03 to 0.15 ng/mL), and higher in the breeding season than the non-breeding season. These results were unexpected, as previous studies using radioimmunoassays found higher DHEA concentrations in the plasma (~0.8 to 1.5 ng/mL) during both breeding and non-breeding seasons (Soma and Wingfield, 2001; Newman, Pradhan and Soma, 2008; Newman and Soma, 2009; Newman et al., 2010, 2013). Nevertheless, the plasma DHEA levels measured here (following derivatization) are similar to those in a recent study also used derivatization and LC-MS/MS (Fokidis et al., 2019). The higher levels of plasma DHEA measured by radioimmunoassays might be the result of antibody cross-reactivity. Therefore, we examined whether several DHEA metabolites, which are structurally similar to DHEA, are present in song sparrow plasma and candidates to cross-react with the anti-DHEA antibodies. We examined 7α-OH-DHEA, 7β-OH-DHEA, 16α-OH-DHEA, and androstenediol. None of these DHEA metabolites were detected in breeding or non-breeding plasma samples. Future studies will examine other DHEA metabolites, such as DHEA fatty acid esters (Tsutsui and Yamazaki, 1995; Soma and Wingfield, 2001).

2.4.3 Local regulation of steroids in the SDMN

The songbird brain is capable of synthesizing sex steroids either de novo from cholesterol or from circulating precursors (Schmidt et al., 2008; Schlinger, 2015). In particular, the male song sparrow brain expresses 3β-HSD, aromatase, 17β-HSD, and 5α-reductase (Soma et al., 2003; Pradhan et al., 2010). In the breeding season, T and 5α-DHT were detected in all 10 brain areas, and interestingly, local levels varied across brain regions. Because the BnST expresses
many androgen receptors (Fuxjager et al., 2010; Wacker et al., 2010), it is surprising that T and 5α-DHT levels are low in this region. It is also noteworthy that androstenedione and 5α-DHT levels are much higher in some regions than in the blood (up to 20-fold higher) similar to a recent study in quail using gas chromatography–mass spectrometry (Liere et al., 2019). CYP17A1 (an enzyme that synthesizes androstenedione) is expressed in the songbird brain (London, Boulter and Schlinger, 2003), with high levels in the VMH and low levels in the LS (similar to androstenedione levels observed here). Thus, we speculate that androstenedione is synthesized locally in the brain. In contrast, T is consistently lower in the brain than in the blood, perhaps because T can be converted in the bird brain to androstenedione, 5α-DHT, and 5β-DHT, as well as 17β-E2 (Balthazart, 1989; Balthazart and Ball, 1998).

In the breeding season, as expected, we observed the highest levels of estrone and 17β-E2 in NCM (approximately 1.4 ng/g combined). NCM is a forebrain area that expresses high levels of aromatase (Saldanha et al., 2000), and 17β-E2 levels in NCM fluctuate during conspecific vocal interactions (Remage-Healey, Maidment and Schlinger, 2008). We also detected estrone and/or 17β-E2 in the POA, AH, BnST, VMH, and TnA of breeding males, and these brain areas express high levels of aromatase in song sparrows (Soma et al., 2003; Wacker et al., 2010). We did not detect much of these estrogens in LS, VTA, CG and Cb, where aromatase levels are lower (Wacker et al., 2010). However, the non-detectable levels of 17β-E2 in BnST are surprising, because of the high aromatase expression in this region. In the BnST, aromatase might be inactive under basal conditions, or 17β-E2 might be rapidly metabolized. In ovariectomized rats, 17β-E2 measurement by LC-MS/MS showed higher levels of 17β-E2 in brain than in serum and differences across brain regions (Li and Gibbs, 2019). These brain regions contain high aromatase activity (Li et al., 2016), and interestingly, serum/brain
differences are eliminated by aromatase inhibition (Li and Gibbs, 2019). We did not detect 17α-estradiol in the brain. No estrogens were detectable in the circulation, and therefore brain estrone and 17β-E₂ are likely neurally synthesized.

Non-breeding subjects did not have detectable androgens or estrogens in the brain, which was unexpected given previous results (Heimovics et al., 2016). In a previous study of captive male song sparrows, in microdissected POA, AH, TnA, and NCM, radioimmunoassays detected DHEA, T, and 17β-E₂ in non-breeding subjects (Heimovics et al., 2016). Similarly, in the non-breeding season, DHEA was measured by radioimmunoassay in gross dissections of brain tissue (Newman and Soma, 2009). As these two studies used radioimmunoassays, it is possible that steroid concentrations were overestimated due to cross-reactivity of the antibodies to other steroids (e.g. as seen with 17β-E₂ immunoassays (Haisenleder et al., 2011)). LC-MS/MS is more specific than immunoassays, which might explain why DHEA, T, and 17β-E₂ are non-detectable in the brains of non-breeding subjects in the present study. Moreover, these data are baseline levels of steroids, as we used minimal song playback for capture. It is possible that an aggressive encounter stimulates neurosteroid synthesis during the non-breeding season (Soma, 2006; Soma et al., 2008; Heimovics et al., 2018; Wingfield et al., 2019).

Progesterone levels showed very different seasonal patterns in the blood and brain. Despite the lack of a seasonal change in circulating progesterone levels, we observed clear seasonal changes in brain progesterone levels. In the POA, AH, VMH, TnA, NCM, and Cb, progesterone levels were higher in the non-breeding season than breeding season. During the non-breeding season, neural progesterone synthesis might provide substrate for neural androgen and estrogen synthesis. In addition, in the POA, AH, TnA, and NCM, progesterone is non-detectable in the breeding season, even though circulating progesterone levels are relatively high.
This could be the result of progesterone binding to corticosteroid-binding globulin (CBG) (Charlier et al., 2009; Vashchenko et al., 2016), which would prevent circulating progesterone from entering the brain. Progesterone regulates aggressive behavior. For example, progesterone administration promotes aggression in male lizards (Weiss and Moore, 2004). In mice, VMH neurons that express progesterone receptor play a key role in inter-male aggression (Yang et al., 2013). Stimulation of these neurons in male mice promotes aggression independent of circulating T (Yang et al., 2017). Here, male song sparrows show elevated levels of progesterone in the VMH, and multiple regions show higher progesterone levels in the non-breeding season than the breeding season, which could support the expression of non-breeding aggression.

Corticosterone levels are several times higher in the blood than in the brain, as reported previously (Newman and Soma, 2009). Corticosterone, like progesterone, binds to CBG (Charlier et al., 2009), and thus passage of circulating corticosterone into the brain is also limited to the free fraction (Mendel, 1989; Hammond, 1995; Breuner and Orchinik, 2002). Interestingly, despite the marked seasonality of corticosterone in the circulation, seasonal differences are less prominent in the brain. This might be the result of seasonal changes in CBG (also lower in the non-breeding season), leading to similar levels of free corticosterone in the circulation across the seasons (Breuner and Orchinik, 2002). This fine regulation of corticosterone passage into the brain allows for greater control of local corticosterone levels and protects the brain against the detrimental effects of excess corticosterone. Taken together, these results clearly indicate that steroid levels are regulated quite differently in the brain and circulation.
2.5 Conclusions

In this study, we measured a panel of 10 steroids in multiple brain regions with excellent spatial resolution and obtained a comprehensive picture of natural seasonal neurosteroid fluctuations in wild animals. Steroid levels are locally regulated within different brain regions and are not a simple reflection of steroid levels in the circulation. The results provide further evidence for neural steroid synthesis in songbirds. Finally, our LC-MS/MS protocol for steroid profiling is applicable to other sample types and species and will be broadly useful for neuroendocrinology studies.
### 2.6 Tables and figures

Table 2.1: Lower limit of quantification in different matrices

<table>
<thead>
<tr>
<th>Steroid</th>
<th>Neat (pg/tube)</th>
<th>Blood (ng/mL)</th>
<th>Plasma (ng/mL)</th>
<th>Brain (ng/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Androstenedione</td>
<td>0.2</td>
<td>0.04</td>
<td>0.02</td>
<td>0.2</td>
</tr>
<tr>
<td>T</td>
<td>0.05</td>
<td>0.01</td>
<td>0.005</td>
<td>0.05</td>
</tr>
<tr>
<td>5α-DHT</td>
<td>0.2</td>
<td>0.04</td>
<td>0.02</td>
<td>0.2</td>
</tr>
<tr>
<td>DHEA</td>
<td>4</td>
<td>0.8</td>
<td>0.4</td>
<td>4</td>
</tr>
<tr>
<td>Estrone</td>
<td>0.1</td>
<td>0.02</td>
<td>0.01</td>
<td>0.1</td>
</tr>
<tr>
<td>17β-E₂</td>
<td>0.2</td>
<td>0.04</td>
<td>0.02</td>
<td>0.2</td>
</tr>
<tr>
<td>17α-estradiol</td>
<td>0.1</td>
<td>0.02</td>
<td>0.01</td>
<td>0.1</td>
</tr>
<tr>
<td>Progesterone</td>
<td>0.05</td>
<td>0.01</td>
<td>0.005</td>
<td>0.05</td>
</tr>
<tr>
<td>Corticosterone</td>
<td>0.1</td>
<td>0.02</td>
<td>0.01</td>
<td>0.1</td>
</tr>
<tr>
<td>Pregnenolone</td>
<td>12.5</td>
<td>2.5</td>
<td>1.25</td>
<td>12.8</td>
</tr>
</tbody>
</table>

Note. Sample amount used was 5µL of blood, 10µL of plasma, and 0.98 mg of brain. Abbreviations: 5α-DHT, 5α-dihydrotestosterone; DHEA, Dehydroepiandrosterone; 17β-E₂, 17β-estradiol; T, Testosterone.
Table 2.2: Scheduled multiple reaction monitoring for LC-MS/MS

<table>
<thead>
<tr>
<th>Steroid</th>
<th>Ion mode</th>
<th>Retention time (min)</th>
<th>Quantifier m/z</th>
<th>Qualifier m/z</th>
</tr>
</thead>
<tbody>
<tr>
<td>Androstenedione</td>
<td>ESI +</td>
<td>7.18</td>
<td>287.2→97.2</td>
<td>287.2→109.1</td>
</tr>
<tr>
<td>T</td>
<td>ESI +</td>
<td>7.99</td>
<td>289.0→97.0</td>
<td>289.0→109.1</td>
</tr>
<tr>
<td>5α-DHT</td>
<td>ESI +</td>
<td>9.67</td>
<td>291.2→255.3</td>
<td>291.2→159.1</td>
</tr>
<tr>
<td>Testosterone-d5</td>
<td>ESI +</td>
<td>7.92</td>
<td>294.0→100.0</td>
<td>-</td>
</tr>
<tr>
<td>DHEA</td>
<td>ESI +</td>
<td>8.54</td>
<td>271.1→253.0</td>
<td>271.1→213.2</td>
</tr>
<tr>
<td>DHEA-d6</td>
<td>ESI +</td>
<td>8.47</td>
<td>277.1→219.2</td>
<td>-</td>
</tr>
<tr>
<td>Estrone</td>
<td>ESI -</td>
<td>7.35</td>
<td>269.0→145.0</td>
<td>269.0→143.0</td>
</tr>
<tr>
<td>17β-E₂</td>
<td>ESI -</td>
<td>7.45</td>
<td>271.0→145.0</td>
<td>271.0→143.0</td>
</tr>
<tr>
<td>17α-estradiol</td>
<td>ESI -</td>
<td>8.04</td>
<td>271.0→145.0</td>
<td>271.0→143.1</td>
</tr>
<tr>
<td>17β-E₂-d4</td>
<td>ESI -</td>
<td>7.39</td>
<td>275.0→147.0</td>
<td>-</td>
</tr>
<tr>
<td>Progesterone</td>
<td>ESI +</td>
<td>10.36</td>
<td>315.2→97.0</td>
<td>315.2→109.1</td>
</tr>
<tr>
<td>Progesterone-d9</td>
<td>ESI +</td>
<td>10.32</td>
<td>324.2→100.0</td>
<td>-</td>
</tr>
<tr>
<td>Corticosterone</td>
<td>ESI +</td>
<td>5.89</td>
<td>347.1→121.1</td>
<td>347.1→91.1</td>
</tr>
<tr>
<td>Corticosterone-d8</td>
<td>ESI +</td>
<td>5.76</td>
<td>355.1→124.9</td>
<td>-</td>
</tr>
<tr>
<td>Pregnenolone</td>
<td>ESI +</td>
<td>10.67</td>
<td>299.1→159.1</td>
<td>299.1→105.1</td>
</tr>
<tr>
<td>Pregnenolone-d4</td>
<td>ESI +</td>
<td>10.65</td>
<td>303.0→159.1</td>
<td>-</td>
</tr>
</tbody>
</table>

Abbreviations: 5α-DHT, 5α-dihydrotestosterone; DHEA, Dehydroepiandrosterone; 17β-E₂, 17β-estradiol; ESI, electrospray ionization; T, Testosterone.
Table 2.3: Assay linearity in different matrices

<table>
<thead>
<tr>
<th>Steroid</th>
<th>Neat Slope</th>
<th>Blood (2-20µL)</th>
<th>Plasma (2-20µL)</th>
<th>Brain (0.5-5 mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Androstenedione</td>
<td>0.810</td>
<td>-</td>
<td>0.844</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>0.997</td>
<td>-</td>
<td>0.992</td>
<td>-</td>
</tr>
<tr>
<td>T</td>
<td>0.835</td>
<td>0.849</td>
<td>0.839</td>
<td>0.865</td>
</tr>
<tr>
<td></td>
<td>0.998</td>
<td>0.999</td>
<td>0.998</td>
<td>0.998</td>
</tr>
<tr>
<td>5α-DHT</td>
<td>0.076</td>
<td>-</td>
<td>0.077†</td>
<td>0.077</td>
</tr>
<tr>
<td></td>
<td>0.999</td>
<td>-</td>
<td>0.943</td>
<td>0.993</td>
</tr>
<tr>
<td>Estrone</td>
<td>0.279</td>
<td>-</td>
<td>-</td>
<td>0.279</td>
</tr>
<tr>
<td></td>
<td>0.998</td>
<td>-</td>
<td>-</td>
<td>0.935</td>
</tr>
<tr>
<td>Progesterone</td>
<td>0.593</td>
<td>0.551</td>
<td>0.555</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>0.999</td>
<td>0.998</td>
<td>0.997</td>
<td>-</td>
</tr>
<tr>
<td>Corticosterone</td>
<td>0.185</td>
<td>0.183</td>
<td>0.182</td>
<td>0.188</td>
</tr>
<tr>
<td></td>
<td>0.997</td>
<td>0.998</td>
<td>0.998</td>
<td>0.999</td>
</tr>
</tbody>
</table>

Note. For validations we used breeding male song sparrow samples. Calculations were based on endogenous steroid levels. When endogenous levels were non-detectable, steroids were omitted from the table (DHEA, 17β-E2, 17α-estradiol, pregnenolone) or dashes were placed in the cells. †linear range 2-10 µL. Abbreviations: 5α-DHT, 5α-dihydrotestosterone; T, Testosterone.
Table 2.4: Assay recovery and precision in different matrices

<table>
<thead>
<tr>
<th>Steroid</th>
<th>Neat recovery %</th>
<th>Blood (5µL) recovery %</th>
<th>Plasma (10µL) recovery %</th>
<th>Brain (1-2 mg) recovery %</th>
<th>Intra-assay variation %</th>
<th>Inter-assay variation %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Androstenedione</td>
<td>120</td>
<td>93</td>
<td>118</td>
<td>91</td>
<td>2.2</td>
<td>4.0</td>
</tr>
<tr>
<td>T</td>
<td>123</td>
<td>100</td>
<td>-</td>
<td>93</td>
<td>1.8</td>
<td>2.0</td>
</tr>
<tr>
<td>5α-DHT</td>
<td>117</td>
<td>-</td>
<td>111</td>
<td>108</td>
<td>5.2</td>
<td>5.1</td>
</tr>
<tr>
<td>DHEA</td>
<td>108</td>
<td>-</td>
<td>-</td>
<td>85</td>
<td>9.8</td>
<td>10.3</td>
</tr>
<tr>
<td>Estrone</td>
<td>116</td>
<td>85</td>
<td>94</td>
<td>93</td>
<td>3.3</td>
<td>3.7</td>
</tr>
<tr>
<td>17β-E2</td>
<td>113</td>
<td>98</td>
<td>107</td>
<td>100</td>
<td>9.1</td>
<td>9.4</td>
</tr>
<tr>
<td>17α-estradiol</td>
<td>126</td>
<td>92</td>
<td>90</td>
<td>92</td>
<td>5.4</td>
<td>7.6</td>
</tr>
<tr>
<td>Progesterone</td>
<td>124</td>
<td>94</td>
<td>-</td>
<td>85</td>
<td>4.2</td>
<td>5.8</td>
</tr>
<tr>
<td>Corticosterone</td>
<td>119</td>
<td>-</td>
<td>-</td>
<td>105</td>
<td>1.8</td>
<td>2.1</td>
</tr>
</tbody>
</table>

Note. Samples were spiked with 0.8 pg of each steroid except for DHEA (8 pg DHEA added). Recovery was not possible to assess when endogenous steroid levels were very high in relation to the spike (e.g. plasma corticosterone) or when the spike was below the lower limit of quantification (in those cases steroids were omitted from the table or dashes were placed in the cells). Abbreviations: 5α-DHT, 5α-dihydrotestosterone; DHEA, Dehydroepiandrosterone; 17β-E2, 17β-estradiol; T, Testosterone.
Table 2.5: Steroid levels in plasma

<table>
<thead>
<tr>
<th>Plasma concentration (ng/mL)</th>
<th>Breeding season</th>
<th>Non-breeding season</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Androstenedione</td>
<td>0.32 ± 0.064</td>
<td>&lt; 0.02</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>T</td>
<td>3.46 ± 0.69</td>
<td>0.015 ± 0.004</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>5α-DHT</td>
<td>0.27 ± 0.047</td>
<td>&lt; 0.02</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>Progesterone</td>
<td>0.53 ± 0.19</td>
<td>0.41 ± 0.17</td>
<td>0.55</td>
</tr>
<tr>
<td>Corticosterone</td>
<td>16.09 ± 2.76</td>
<td>5.07 ± 0.78</td>
<td>0.001</td>
</tr>
</tbody>
</table>

Data are mean ± SEM. n = 10/season. Abbreviations: 5α-DHT, 5α-dihydrotestosterone; T, Testosterone.
**Table 2.6: Brain to blood ratio of androgens in the breeding season**

<table>
<thead>
<tr>
<th>Brain area</th>
<th>Androstenedione</th>
<th>T</th>
<th>5α-DHT</th>
</tr>
</thead>
<tbody>
<tr>
<td>POA</td>
<td>3.0 ± 1.4</td>
<td>0.3 ± 0.04</td>
<td>19.9 ± 2.9</td>
</tr>
<tr>
<td>AH</td>
<td>2.7 ± 1.3</td>
<td>0.4 ± 0.03</td>
<td>17.5 ± 2.0</td>
</tr>
<tr>
<td>LS</td>
<td>nd</td>
<td>0.4 ± 0.03</td>
<td>14.1 ± 1.5</td>
</tr>
<tr>
<td>BnST</td>
<td>3.2 ± 1.8</td>
<td>0.2 ± 0.04</td>
<td>6.1 ± 0.7</td>
</tr>
<tr>
<td>VMH</td>
<td>6.1 ± 3.0</td>
<td>0.4 ± 0.04</td>
<td>16.0 ± 1.9</td>
</tr>
<tr>
<td>VTA</td>
<td>3.7 ± 1.2</td>
<td>0.7 ± 0.04</td>
<td>18.5 ± 2.4</td>
</tr>
<tr>
<td>CG</td>
<td>2.8 ± 0.9</td>
<td>0.7 ± 0.05</td>
<td>17.3 ± 2.2</td>
</tr>
<tr>
<td>NCM</td>
<td>5.4 ± 2.3</td>
<td>0.5 ± 0.05</td>
<td>11.8 ± 1.7</td>
</tr>
<tr>
<td>TnA</td>
<td>3.7 ± 1.9</td>
<td>0.4 ± 0.03</td>
<td>18.5 ± 2.9</td>
</tr>
<tr>
<td>Cb</td>
<td>1.2 ± 0.4</td>
<td>0.6 ± 0.06</td>
<td>11.8 ± 1.5</td>
</tr>
</tbody>
</table>

Data are mean ± SEM. n = 9-10. Abbreviations: nd, non-detectable; 5α-DHT, 5α-dihydrotestosterone; T, Testosterone; POA, Preoptic area; AH, Anterior hypothalamus; LS, Lateral septum; BnST, Bed nucleus of the stria terminalis; VMH, Ventromedial hypothalamus; TnA, Nucleus taeniae of the amygdala; NCM, Caudomedial nidopallium; VTA, Ventral tegmental area; CG, Central gray; Cb, Cerebellum.
Figure 2.1: Simplified steroidogenesis pathway.

The steroids measured are underlined. Abbreviations: 3β-HSD, 3β-hydroxysteroid dehydrogenase/isomerase; 17β-HSD, 17β-hydroxysteroid dehydrogenase.
Figure 2.2: Representative punch locations in the song sparrow brain.

Coronal sections are presented from rostral to caudal. 1 mm punches are represented by circles (approximately to scale). Figures adapted from a zebra finch brain atlas (Nixdorf-Bergweiler & Bischof, 2007). Abbreviations: preoptic area (POA), lateral septum (LS), bed nucleus of the stria terminalis (BnST), anterior commissure (CoA), anterior hypothalamus (AH), ventromedial hypothalamus (VMH), tractus occipito-mesencephalicus (OM), ventral tegmental area (VTA), posterior commissure (PC), central gray (CG), caudomedial nidopallium (NCM), nucleus taeniae of the amygdala (TnA), and cerebellum (Cb).
Figure 2.3: Representative chromatograms for the quantifier ions of all the analytes.

Shown are data from certified reference steroids (100 pg for all steroids except DHEA, 1000 pg for DHEA). Upper panel shows the steroids measured using positive electrospray ionization mode, and the bottom panel shows the steroids measured using negative electrospray ionization mode. Intensity in counts per second (cps).
Figure 2.4: Circulating androgen levels in blood of wild male song sparrows.

(A-C) Seasonal variation in blood levels (ng/mL) of (A) androstenedione, (B) testosterone, and (C) 5α-dihydrotestosterone. Values are expressed as mean ± SEM. n = 9-10 per group. ****p < 0.0001. (D-E) Correlations between blood androgen levels during the breeding season only. (D) Blood levels of androstenedione and testosterone ($r^2 = 0.5359$, $p = 0.0249$), and (E) blood levels of testosterone and 5α-dihydrotestosterone ($r^2 = 0.8545$, $p = 0.0001$). n = 9-10 per group. Abbreviations: 5α-DHT, 5α-dihydrotestosterone; nd, non-detectable.
Figure 2.5: Androgen levels in the brain of breeding male song sparrows.

Bar graphs represent concentrations of (A) androstenedione, (B) testosterone, and (C) 5α-dihydrotestosterone. Values are expressed as mean ± SEM, n = 10 per group. Dashed lines indicate mean blood level of each steroid during the breeding season. Abbreviations: 5α-DHT, 5α-dihydrotestosterone; nd, non-detectable.
Figure 2.6: Estrogen levels in the brain of breeding male song sparrows.

Bar graphs represent concentrations of (A) estrone and (B) 17β-estradiol. Values are expressed as mean ± SEM, n = 10 per group. Abbreviations: nd, non-detectable. Note that circulating estrone and 17β-estradiol were not detected. 17α-estradiol was not detected in blood or brain.
Figure 2.7: Seasonal variations in progesterone and corticosterone in the blood and brain in wild male song sparrows.

Bar graphs represent concentrations of (A) progesterone and (B) corticosterone. Values are expressed as mean ± SEM, n = 10 per group. *p < 0.05; **p < 0.01; ***p < 0.001. Abbreviations: nd, non-detectable.
3.1 Introduction

The brain can locally produce steroids, either de novo from cholesterol or from conversion of circulating precursors (Schmidt et al., 2008; Schlinger, 2015). The brain expresses all the necessary enzymes for steroid synthesis (Tsutsui, 2011) in a region-specific manner (Soma et al., 2003; Wacker et al., 2010). Brain-derived steroids, known as neurosteroids, were first characterized in rodents (Baulieu, 1991; Mellon, Griffin and Compagnone, 2001; Hojo and Kawato, 2018), and later in other vertebrates such as birds (Schmidt et al., 2008; Tsutsui, 2011; Schlinger, 2015). In particular, estrogens are produced from androgens by the aromatase enzyme, which exhibits high activity in specific brain regions (Naftolin and Ryan, 1975). Bioactive estrogenic metabolites such as catechol estrogens are also locally synthesized within the brain, although their functions are less clear (Fowke et al., 2003; Denver, Khan, Stasinopoulos, Church, N. Z. Homer, et al., 2019).

High neuroestrogen production occurs in songbirds, and seasonal changes in local estrogen production affect aggressive behavior. The song sparrow, Melospiza melodia, which is common along the Pacific Coast of North America, is an excellent model for investigating neurosteroid production and the regulation of territorial aggression (Patten and Pruett, 2009; Wacker, 2019). Males exhibit aggression during breeding (spring) and non-breeding (autumn) seasons but not during molt (late summer) (Wingfield and Hahn, 1994; Wingfield and Soma, 2002). Circulating levels of testosterone are high during the breeding season but very low during
the non-breeding season. In the non-breeding season, castration does not reduce aggression, but inhibition of aromatase does reduce aggression (Wingfield, 1994; Soma et al., 2000; Soma, Tramontin and Wingfield, 2000). Administration of 17β-estradiol (17β-E2) increases aggression in non-breeding males (Heimovics, Ferris and Soma, 2015). Social behavior, including aggression, is regulated by the social behavior network (SBN). The SBN expresses steroidogenic enzymes as well as sex steroid receptors, indicating that this circuit is both steroid-synthetic and sensitive (Newman, 1999; Goodson, 2005). In song sparrows, aromatase is highly expressed in the SBN and varies seasonally (Soma et al., 2003; Wacker et al., 2010). Altogether, these data suggest that seasonal changes in neuroestrogen synthesis contribute to seasonal changes in territorial aggression in song sparrows (Jalabert et al., 2018; Quintana et al., 2021). To measure estrogens in specific regions of the SBN, a highly specific and sensitive method is required.

Estrogens can be measured by liquid chromatography tandem mass spectrometry (LC-MS/MS), a highly specific and sensitive technique that allows for simultaneous measurement of multiple analytes. Immunoassays can suffer from cross-reactivity with structurally similar steroids (Faqehi et al., 2016). Estrogen measurement with mass spectrometry can be impeded by poor ionization efficiency, but this problem can be ameliorated by derivatization, which adds an easily ionized group or charged moiety to the analyte of interest (Faqehi et al., 2016; Denver, Khan, Homer, et al., 2019). Dansyl chloride is a commonly used derivatization reagent for estrogens. However, with dansyl chloride, the product ions are not analyte-specific because they are solely produced from the dansyl chloride moiety (Xu and Spink, 2008; Li and Franke, 2015). In addition, the sensitivity increase with dansyl chloride is not sufficient to measure estrogens in microdissected brain (C.J. and K.K.S., unpub. results). In contrast, 1,2-dimethylimidazole-5-sulfonyl-chloride (DMIS) derivatization is estrogen-specific, generates analyte-specific product
ions, produces lower background values, and yields greater sensitivity in measuring 17β-E2 and estrone (E1).

Here, we developed a method to measure several estrogens simultaneously with high
specificity and sensitivity, in microdissected brain regions. We derivatized estrogens with DMIS.
Using LC-MS/MS, we initially aimed to measure a panel of eight estrogens: E1, 17β-E2, 17α-
estradiol (17α-E2), estriol (E3), 2-hydroxyestradiol (2OH-E2), 4-hydroxyestradiol (4OH-E2), 2-
methoxyestradiol (2Me-E2), and 4-methoxyestradiol (4Me-E2) (Figure 3.1). The method was
successfully validated for E1, 17β-E2, 17α-E2, and E3 in whole blood (hereafter “blood”), plasma,
and brain of song sparrows. In contrast, the method was not sufficient for quantitation of 2OH-
E2, 4OH-E2, 2Me-E2, and 4Me-E2, primarily because of matrix effects. To achieve high spatial
resolution, we microdissected 11 regions. We then examined seasonal changes of estrogens in
blood, plasma, and microdissected brain regions in free-living male song sparrows.

3.2 Materials and methods

3.2.1 Field procedures

Song sparrows are widespread and abundant throughout North America (especially near
Vancouver, BC) and their conservation status is of least concern, according to the UICN red list.
Free-living adult male song sparrows were captured in the non-breeding season (October 26th to
November 8th, 2018, n = 11) and breeding season (April 9th to April 24th, 2019, n = 10).
Another 4 animals were captured for method validation. Subjects were captured near Vancouver,
BC using a mist net and conspecific song playback for a maximum of 5 min (breeding: 1.6 ± 0.5
min, non-breeding: 1.6 ± 0.6 min; p = 0.51), to avoid effects of song playback on steroid levels.
Immediately after capture, the subject was rapidly and deeply anesthetized with isoflurane and
then rapidly decapitated. There was a maximum of 3 min between capture and euthanasia (breeding: $2.6 \pm 0.2$ min, non-breeding: $2.2 \pm 0.3$ min; $p = 0.23$), to avoid effects of handling on steroid levels. The brain was immediately collected and snap frozen on powdered dry ice. Trunk blood was collected in heparinized microhematocrit tubes (Fisher Scientific) that were kept on ice packs until return to the laboratory within 5 h.

Once in the laboratory, blood was divided into 2 aliquots. One half of the blood sample was frozen. The other half of the blood sample was centrifuged, and then plasma was collected and frozen. All samples were stored at $-70^\circ$C until steroids were extracted. Blood and plasma samples were used to measure circulating levels of estrogens. Plasma overestimates circulating steroid levels, and therefore blood was used as a more accurate estimate of circulating steroid levels and to compare to brain steroid levels (Taves et al., 2010, 2011, 2015).

All procedures were in compliance with the Canadian Council on Animal Care and protocols were approved by the Canadian Wildlife Service and the [Author University] Animal Care Committee.

### 3.2.2 Brain Microdissection

To microdissect brain tissue, the Palkovits punch technique (Palkovits, 1973) was used as before (Charlier et al., 2011; Heimovics et al., 2016; Jalabert, Ma and Soma, 2021). Tissue was collected from 11 brain regions (Figure 3.2), including those in the social behavior network: nucleus accumbens (NAc), preoptic area (POA), anterior hypothalamus (AH), lateral septum (LS), bed nucleus of the stria terminalis (BnST), ventromedial hypothalamus (VMH), ventral tegmental area (VTA), central grey (CG), caudomedial nidopallium (NCM), nucleus taeniae of the amygdala (TnA) (homolog of the mammalian medial amygdala), and cerebellum (Cb).
Brains were sectioned in the coronal plane at 300 µm using a MicroHM525 cryostatic microtome (Thermo Fisher Scientific Inc., Waltham, MA) at -12°C while using a plane of sectioning that closely matched a zebra finch brain atlas (Nixdorf-Bergweiler and Bischof, 2007). Coronal sections were then mounted onto Superfrost Plus microscope slides (Thermo Fisher Scientific) and examined to identify brain regions of interest and major neuroanatomical landmarks.

Frozen sections were microdissected using a stainless-steel biopsy punch tool (Integra Miltex biopsy punch tool, 1 mm diameter, tissue wet weight 0.245 mg per punch). One punch (centered at the midline for one section) was collected containing the NAc, ventral to the area X. Four punches (two per side for two serial sections) were collected containing the POA in two sections caudal to the last section containing the tractus septopalliomesencephalicus (TrSM) and rostral to the AH. Four punches (two per side for two serial sections) were collected for the AH, immediately caudal to POA sections and ventral to the anterior commissure (CoA). Four punches (two per side for two serial sections) were collected for the LS and BnST, dorsal to the CoA. The LS was collected medial to the lateral ventricles, and the BnST was collected at the tip of each lateral ventricle. Four punches (two per side for two serial sections) containing VMH were collected ventral to the AH. Four punches (two per side for two serial sections) containing the VTA were collected ventrolateral to the oculomotor nerve. Four punches (two per side for two serial sections) containing the CG were collected ventral to the posterior commissure. Six punches (two per side for three serial sections) containing the NCM were collected starting at the last appearance of the CoA and tractus occipito-mesencephalicus path from the ventromedial telencephalon. Six punches (two per side for three serial sections) containing the TnA were collected immediately caudal to the disappearance of the CoA and tractus occipito-mesencephalicus. Six punches (two at the midline for three serial sections) containing the Cb
were collected starting at its first appearance. Punches were expelled into 2-mL polypropylene tubes (Sarstedt AG & Co, Numbrecht Germany, 72.694.007) that each contained five zirconium ceramic oxide beads (1.4-mm diameter). Punches were then stored at -70 °C until further processing.

3.2.3 Reagents

High Performance Liquid Chromatography (HPLC)-grade acetone, acetonitrile, hexane, and methanol were from Fisher Chemical. Here we used 1,2-dimethylimidazole-5-sulfonyl-chloride (DMIS) for derivatization (Figure 3.3). Note that we did not use the isomer 1,2-dimethylimidazole-4-sulfonyl chloride, which has been used for estrogen derivatization, but the fragmentation is not analyte-specific (Xu and Spink, 2007). Dry DMIS (Apollo Scientific, Stockport United Kingdom, Lot number: AS478881, CAS number: 849351-92-4) was stored at 4°C under nitrogen gas and protected from light and moisture. Then, dry DMIS was aliquoted and stored at 4°C (protected from light and moisture but not under nitrogen gas) for up to 12 months (time of storage did not affect dry DMIS stability). Acetone was added to individual aliquots of DMIS on the day of derivatization to prepare fresh DMIS solution at 1 mg/mL. Sodium bicarbonate buffer (50mM, pH 10.5) was prepared in Milli-Q water.

Stock solutions were prepared in HPLC-grade methanol. Certified reference standards of E1, 17β-E2, and E3 were obtained from Cerilliant. 17α-E2, 2Me-E2, 4Me-E2, and 4OH-E2 were obtained from Steraloids. Calibration curves were prepared in 50% methanol. The calibration curve ranged from 0.01 to 20 pg per tube for E1, 17β-E2, 17α-E2, E3, 2Me-E2, and 4Me-E2, and from 0.1 to 200 pg per tube for 4OH-E2. The catechol estrogens, 2OH-E2 and 4OH-E2, displayed the same fragmentation patterns and retention times after DMIS derivatization and thus were
indistinguishable using our assay. As a result, we only included 4OH-E2 in our calibration curve. 17α-E2 showed the same fragmentation pattern as 17β-E2 and the retention time only differed by approximately 0.14 min (causing the peaks to overlap). Therefore, we included 17α-E2 in a separate calibration curve. Internal standard (IS) stock solution of 17β-Estradiol-2,4,16,16-d4 (17β-E2-d4, C/D/N Isotopes, Canada, catalog number: D-4318, CAS number: 66789-03-5) was prepared in methanol and further diluted with 50% methanol to a final working solution of 40 pg/mL.

3.2.4 Steroid extraction

Steroids were extracted from brain tissue (sample amount detailed above for each brain region), blood (20 µL), and plasma (20 µL) similar to before (Jalabert, Ma and Soma, 2021). One mL of acetonitrile was added to all samples, and 50 µL (i.e. 2 pg) of IS 17β-E2-d4 was added to all samples except “double blanks.” Samples were then homogenized using a bead mill homogenizer (Omni International Inc., Kennesaw, GA, USA) at 4 m/s for 30 sec. Samples were then centrifuged at 16,100 g for 5 min, and 1 mL of supernatant was taken from each sample and placed into a borosilicate glass culture tube (12x75mm) that had been cleaned with methanol. After the addition of 500 µl of hexane, tubes were vortexed and centrifuged at 3200 g for 2 min. Hexane was removed and discarded, and extracts were dried at 60°C for 45 min in a vacuum centrifuge (ThermoElectron SPD111V; Thermo Fisher Scientific). Calibration curves, quality controls (QCs), blanks, and double blanks were prepared alongside samples. Underivatized standards, in which acetone was added without DMIS, were prepared in parallel to measure any underivatized estrogens and calculate derivatization reaction efficiency (94 to 100% for all estrogens).
3.2.5 DMIS derivatization

Derivatization was based on previous studies (Keski-Rahkonen et al., 2015; Handelsman et al., 2020). Here, the protocol was slightly modified to reduce reagent evaporation. Dried extracts were immersed in an ice bath, then samples were reconstituted with 30 µL of sodium bicarbonate buffer (50 mM, pH 10.5), briefly vortexed, and 20 µL of 1 mg/mL DMIS in acetone was added. Samples were then vortexed and centrifuged at 3200 g for 1 min before being transferred to glass LC-MS vial inserts placed in LC-MS vials (Agilent, Santa Clara, CA, USA). Vials were capped to prevent evaporation during incubation for 15 min at 60°C. This was followed by a cooling period of 15 min at 4°C. Samples were centrifuged at 3200 g for 1 min, and then stored at -20°C for no more than 24 h before steroid analysis.

3.2.6 Steroid analysis by LC-MS/MS

Steroids were quantified using a Sciex 6500 Qtrap UHPLC-MS/MS system (Jalabert, Ma and Soma, 2021). Samples were transferred into a refrigerated autoinjector (15°C). Then, 45 µL from each sample were injected into a Nexera X2 UHPLC system (Shimadzu Corp., Kyoto, Japan), passed through a KrudKatcher ULTRA HPLC In-Line Filter (Phenomenex, Torrance, CA) and then an Agilent 120 HPH C18 guard column (2.1 mm), and separated on an Agilent 120 HPH C18 column (2.1 x 50 mm; 2.7 µm; at 40°C) using 0.1 mM ammonium fluoride in MilliQ water as mobile phase A (MPA) and methanol as mobile phase B (MPB). The flow rate was 0.4 mL/min. During loading, MPB was at 10% for 1.6 min, and from 1.6 to 4 min the gradient profile was at 42% MPB, which was ramped up to 60% MPB until 9.4 min. From 9.4 to 11.9 min the gradient was ramped from 60% to 98% MPB until 13.4 min. Finally, a column wash was
performed from 11.9 to 13.4 min at 98% MPB. The MPB was then returned to starting conditions of 10% MPB for 1.5 min. Total run time was 14.9 min. The needle was rinsed externally with 100% isopropanol before and after each sample injection.

We used two multiple reaction monitoring (MRM) transitions for each estrogen and one MRM transition for the deuterated IS (Table 3.1). Steroid concentrations were acquired on a Sciex 6500 Qtrap triple quadrupole tandem mass spectrometer (Sciex LLC, Framingham, MA) in positive electrospray ionization mode for all derivatized estrogens and negative electrospray ionization mode for underivatized estrogens (Table 3.1). All water blanks were below the lowest standard on the calibration curves.

3.2.7 Stability of internal standard

Deuterated IS can potentially experience hydrogen-deuterium exchange (Kwok et al., 2010; Viljanto et al., 2018), so we tested for possible alterations of 17β-E2-d4 caused by the derivatization procedure. We compared the mass spectra of 17β-E2-d4 directly from the stock solution, after sham derivatization (resuspension in buffer and acetone followed by incubation for 15 min at 60°C, without DMIS), or after derivatization (resuspension in buffer and DMIS in acetone followed by incubation for 15 min at 60°C). In addition, we tested for effects of heating on the IS by comparing the mass spectra of 17β-E2-d4 resuspended in buffer and acetone either incubated for 15 min at 60°C or not incubated. We also examined unlabeled 17β-E2 either directly from the stock solution or after derivatization (resuspension in buffer and DMIS in acetone followed by incubation for 15 min at 60°C). All samples were prepared at 10 µg/mL for the infusion at 7 µL/min using a syringe pump and all other LC–MS/MS parameters were identical to those described in the previous section.
For non-derivatized samples, we evaluated quadrupole 1 (Q1) ion of $^{17}\beta$-E$_2$ (271 m/z) and $^{17}\beta$-E$_2$-d$_4$ (275 m/z), and because the IS has 4 deuterium atoms and possible hydrogen-deuterium exchange, we scanned the mass spectra range from 270 to 276 for other Q1 ions (m/z, 272, 273, and 274). We also analyzed the following MRM (m/z) for non-derivatized samples: 271→145, 272→145, 272→146, 273→145, 273→146, 274→146, 274→147, and 275→147. For derivatized samples, we evaluated Q1 ion of $^{17}\beta$-E$_2$-DMIS (431 m/z) and $^{17}\beta$-E$_2$-d$_4$-DMIS (435 m/z) and the mass spectra range from 430 to 436. We also analyzed the following MRM (m/z) for derivatized samples: 431→367, 432→368, 433→369, 434→370, and 435→371.

3.2.8 Assay accuracy and precision

Assay accuracy was determined by measuring QCs containing known amounts of estrogens (0.5 and 2 pg for all estrogens, except for the catechol estrogens where 5 and 20 pg were used) in neat solution. Precision was determined from both intra-assay and inter-assay variation by calculating the coefficient of variation of QCs. The acceptance criteria aligned with FDA style guidelines.

3.2.9 Stability of derivatized analytes

Stability of derivatized analytes was assessed by measuring a 10 pg standard of E$_1$, $^{17}\beta$-E$_2$, $^{17}\alpha$-E$_2$, E$_3$, 2Me-E$_2$, and 4Me-E$_2$ and 20 pg of 4OH-E$_2$ at different storage times and temperatures. Samples were derivatized on different days, so that all samples could be injected on the same day, to ensure that LC-MS/MS conditions were the same for all samples. One set (n=3) of standards was injected immediately after derivatization. The other sets of standards
were injected after 24 hours at 15°C, as well as after 1, 4, 8 and 31 d at -20°C or -70°C (n=3 per set).

3.2.10 Matrix effects and recoveries

The protocol was validated in song sparrow brain, blood, and plasma. First, matrix effects were tested by creating pools and then performing serial dilutions (0.5, 1, 2, and 4 mg for brain tissue, and 2, 5, 10, and 20 µL for blood or plasma) to assess linearity and parallelism to the calibration curves. Second, we compared the peak areas for the IS in the three matrices and neat solution. Differences in IS peak area of less than 20% were considered acceptable. Third, recovery was assessed by creating a pool that was divided in two, one was spiked with a known amount of steroid and the other one was unspiked. We calculated the difference in steroid concentration between those two and compared with the spike in neat solution. Recoveries were evaluated in blood, plasma, and brain at the sample amounts described above.

3.2.11 Statistical analysis

A value was considered non-detectable if it was below the lowest standard on the calibration curve. When 20% or more of the samples in a group (blood or brain region) were detectable, then the non-detectable values were estimated via quantile regression imputation of left-censored missing data using MetImp web tool (Wei, Wang, Jia, et al., 2018; Wei, Wang, Su, et al., 2018; Tobiansky et al., 2021). Data were imputed for each season and each estrogen independently, and imputed values were between 0 and the lowest standard on the calibration curve. When less than 20% of the samples in a group (blood or brain region) were detectable, then imputations were not performed, data were not analyzed statistically, and data are only
reported in the text. To compare steroid levels in brain and blood, we assumed that 1 mL of blood weighs 1 g (Taves et al., 2011; Tobiansky et al., 2020).

Statistics were conducted using GraphPad Prism version 9.02 (GraphPad Software, La Jolla, CA). When necessary, data were log transformed prior to analysis. Regional differences in estrogen levels were analyzed by repeated measures one-way analysis of variance (ANOVA). ANOVAs were followed by Tukey multiple comparison tests and corrected p values are shown. Significance criterion was set at p ≤ 0.05. Graphs show the mean ± standard error of the mean (SEM) and are presented using the non-transformed data.

3.3 Results

We aimed to measure a panel of eight estrogens with DMIS derivatization and succeeded in measuring four estrogens (E1, 17β-E2, 17α-E2, and E3). For 2OH-E2, 4OH-E2, 2Me-E2 and 4Me-E2, there were various problems (mentioned below).

3.3.1 Specificity

For E1, 17β-E2, 17α-E2, and E3, the assay showed high specificity after the optimization of the liquid chromatography and scheduled MRM transitions (Table 3.1).

The isomers 2OH-E2 and 4OH-E2 were indistinguishable because of their identical retention times and identical quantifier and qualifier transitions (Table 3.1). As a result, we only included 4OH-E2 in the calibration curve and QCs.
3.3.2 Sensitivity

Derivatization with DMIS greatly improved sensitivity of estrogen measurement. Using 1 pg of each estrogen in neat solution, we observed increased peak areas of analytes when derivatized with DMIS (Figure 3.4B) compared to an assay without DMIS (Figure 3.4A). The calibration curves were linear, even at the low range, demonstrating excellent assay sensitivity (Figure 3.6).

The lower limit of quantification (LLOQ) was enhanced by DMIS derivatization for all estrogens (Table 3.2). After DMIS derivatization, 17β-E2 and E3 showed a 10-fold improvement in sensitivity, and the LLOQ went from 0.2 pg/tube to 0.02 pg/tube. For 17α-E2 and E1, the LLOQ went from 0.1 pg/tube to 0.02 pg/tube (Table 3.2).

Due to their unstable nature, 2OH-E2 and 4OH-E2 were not detected without derivatization; however following derivatization, the LLOQ was 2 pg/tube. For 2Me-E2 and 4Me-E2, the LLOQ went from 0.5 pg/tube to 0.05 pg/tube and 0.2 pg/tube respectively (Table 3.2).

3.3.3 Stability of internal standard

The Q1 and MRM of 17β-E2-d4 (directly from stock solution, after sham derivatization, and after DMIS derivatization) and 17β-E2 (directly from stock solution and after DMIS derivatization) are presented in Figure 5. The 17β-E2 mass spectrum is characterized by the presence of abundant 271 m/z deprotonated molecule (Figure 3.5 A and B). The mass spectra of 17β-E2-d4 directly from stock solution (Figure 3.5 E and F) and after sham derivatization (Figure 3.5 G and H) showed the presence of abundant 275 m/z molecule, indicating that deuterium loss did not occur. Derivatized 17β-E2 mass spectrum showed abundant 431 m/z (Figure 3.5 C and D)
and, most importantly, derivatized 17β-E2-d4 was characterized by abundant 435 m/z (Figure 3.5 I and J). In addition, we did not detect an effect of heating 17β-E2-d4 (data not shown). Taken together, the data indicate the stability of the deuterated IS under the present conditions for derivatization.

3.3.4 Accuracy and precision

Accuracy and precision were measured using QCs at two amounts of estrogens in neat solution (Table 3.3). Accuracies were approximately 100% for all estrogens at both amounts (Table 3.3).

Precision was measured as the coefficient of variation for QC replicates at both amounts. The intra-assay variation was acceptable in all cases (Table 3.3). For the inter-assay variation, the QCs were measured across multiple assays and acceptable for E1 (11%), 17β-E2 (7%), 17α-E2 (8%), and E3 (6%).

Precision was lower for catechol and methoxy estrogens: 4OH-E2 (24%), 2Me-E2 (26%), and 4Me-E2 (14%).

3.3.5 Stability of derivatized analytes

Stability of seven derivatized estrogens were measured at varying temperatures and durations of storage. Storage temperatures were 15°C (autosampler temperature), -20°C, and -70°C. Durations of storage were 0, 1, 4, 8, and 31 d. Analyte/IS area ratios were expressed relative to time zero (T0), in which injection into the LC-MS/MS occurred immediately following derivatization.
All derivatized estrogens were unaffected by storage in the autosampler at 15°C for 1 d. Moreover, derivatized E1, 17β-E2, 17α-E2, and E3 were unaffected by storage up to 31 d at -20°C or -70°C (Table 3.5). These data indicate that the DMIS derivatives of E1, 17β-E2, 17α-E2, and E3 are stable under normal laboratory operating conditions.

In contrast, derivatized catechol and methoxy estrogens were less stable. Derivatized 4OH-E2, 2Me-E2, and 4Me-E2 declined after 1 d at -20°C or -70°C (Table 3.5). Similarly, derivatized 4OH-E2 decreased after 4 d at -20°C or -70°C (Table 3.5). Derivatized 2Me-E2 and 4Me-E2 decreased after 8 and 31 d at -20°C or -70°C (Table 3.5).

3.3.6 Method validation in brain matrix

First, matrix effects were assessed by creating a 60 mg pool of homogenized song sparrow forebrain tissue. This pool of brain homogenate was then spiked with estrogens and serial diluted (4, 2, 1, and 0.5 mg per tube) to evaluate linearity. The slope of each estrogen in neat solution was compared to its slope in brain tissue, to determine the extent of matrix interference. Differences in slope were measured for E1 (7%), 17β-E2 (2%), E3 (7%), 4OH-E2 (2%), 2Me-E2 (1%) and 4Me-E2 (19%) and were satisfactory (Table 3.4). Second, the IS peak area in brain tissue was compared to the IS peak area in neat solution and ranged from 111-118% across brain tissue amounts (0.5 - 4 mg). Third, recoveries were assessed by subtracting unspiked sample values from spiked sample values from the same pool and dividing by the amount of estrogen added. Recoveries were calculated across brain tissue amounts (0.5 - 4 mg) and were acceptable for E1 (102%), 17β-E2 (102%), and E3 (93%). Recoveries were high and not acceptable for 4OH-E2 (613%), 2Me-E2 (214%), and 4Me-E2 (247%) (Table 3.4), suggesting matrix effects with brain tissue for 4OH-E2, 2Me-E2, and 4Me-E2.
3.3.7 Method validation in blood matrix

First, matrix effects were assessed by creating a 266 µL pool of song sparrow blood. This pool of blood was then spiked and serial diluted (20, 10, 5, and 2 µL per tube) to evaluate linearity. The slope of each estrogen in neat solution was compared to its slope in blood. Differences in slope were measured for E1 (3%), 17β-E2 (1%), E3 (5%), 2Me-E2 (3%), and 4Me-E2 (3%) and were satisfactory (Table 3.4). However, 4OH-E2 was not detectable when spiked in blood (Table 3.4). Second, the IS peak area in blood was compared to the IS peak area in neat solution and ranged from 85-115% across blood volumes (2-20 µL). Third, recoveries were assessed by subtracting unspiked sample values from spiked sample values from the same pool and dividing by the amount of estrogen added. Recoveries were calculated across blood volumes (2-20 µL) and were acceptable for E1 (90%), 17β-E2 (94%), and E3 (92%). Recoveries were high and not acceptable for 2Me-E2 (295%) and 4Me-E2 (200%) (Table 3.4), suggesting matrix effects with blood for 2Me-E2 and 4Me-E2.

3.3.8 Method validation in plasma matrix

First, matrix effects were assessed by creating a 266 µL pool of song sparrow plasma. This pool of plasma was then spiked and serial diluted (20, 10, 5, and 2 µL per tube) to evaluate linearity. The slope of each estrogen in neat solution was compared to its slope in plasma. Differences in slope were measured for E1 (0%), 17β-E2 (6%), E3 (1%), 4OH-E2 (14%), 2Me-E2 (1%), and 4Me-E2 (2%) and were satisfactory (Table 3.4). Second, the IS peak area in plasma was compared to IS peak area in neat solution and ranged from 80-108% across plasma volumes (2-20 µL). Third, recoveries were assessed by subtracting unspiked sample values from spiked
sample values from the same pool and dividing by the amount of estrogen added. Recoveries were calculated across plasma volumes (2-20 µL) and were acceptable for E1 (97%), 17β-E2 (108%), and E3 (102%). Recoveries were high and not acceptable for 4OH-E2 (98%), 2Me-E2 (291%), and 4Me-E2 (161%) (Table 3.4), suggesting matrix effects with plasma for 4OH-E2, 2Me-E2 and 4Me-E2.

3.3.9 Estrogen levels in microdissected brain regions

We examined 11 brain regions in subjects from two seasons (n=10-11 subjects per season). In non-breeding males, the only estrogen detected in the brain was 17β-E2 in the NCM (14.8 ± 0.9 pg/g).

In contrast, in breeding males, nine brain regions had detectable levels of E1 (Figure 3.7) and ten brain regions had detectable levels of 17β-E2 (Figure 3.7). The Cb had detectable 17β-E2 but not E1. Both E1 and 17β-E2 were non-detectable in the NAc (Figure 3.7). In breeding males, E1 and 17β-E2 levels showed similar patterns across brain regions, with highest levels in NCM (Figure 3.7). 17α-E2 and E3 were non-detectable in the brain of breeding males. Although there were matrix effects with brain tissue, we can very tentatively suggest that 4OH-E2 (LLOQ 2 ng/g), 2Me-E2 (LLOQ 0.05 ng/g), and 4Me-E2 (LLOQ 0.02 ng/g) were non-detectable in the brain of breeding males.

To compare E1 levels across blood and brain regions in breeding males, a one-way repeated measures ANOVA was conducted. E1 levels showed a significant effect of sample type (blood or brain region) (F9,81 = 113.6, P < 0.0001) (Figure 3.7A). Post-hoc comparisons revealed that E1 levels were higher in NCM than other brain regions (all P < 0.0001) except the AH. No differences in E1 levels were found among POA, AH, VMH, TnA; nor among POA, VMH, and
Lastly, no differences were found in E1 levels among LS, VTA, and CG. E1 levels were lower in blood than in POA, AH, LS, BnST, VMH, VTA, CG, NCM, and TnA.

For 17β-E2 levels in breeding males, there was a significant effect of sample type ($F_{10,90} = 89.10 \ P < 0.0001$) (Figure 3.7B). Post-hoc comparisons revealed that 17β-E2 levels were higher in NCM than other brain regions (all $P < 0.0001$) except POA and AH. No differences in 17β-E2 concentrations were found among VTA, CG, Cb, LS and blood; nor between TnA, VMH and POA. 17β-E2 levels were lower in blood than in POA, AH, BnST, VMH, NCM, and TnA.

### 3.3.10 Estrogen levels in circulation

In the non-breeding season, no estrogens were detectable in the blood or plasma ($n = 11$). In the breeding season, E1 was detectable in 50% of blood samples, and 17β-E2 was detectable in 60% of blood samples ($n = 10$). In breeding males, blood E1 level was $2.8 \pm 0.5$ pg/mL, and blood 17β-E2 level was $4.1 \pm 0.7$ pg/mL (Figure 3.7). In breeding males, E1 and 17β-E2 were detectable in 70% of plasma samples ($n = 10$). Plasma E1 level was $3.7 \pm 0.5$ pg/mL, and plasma 17β-E2 level was $4.5 \pm 0.7$ pg/mL. 17α-E2 and E3 were non-detectable in the blood and plasma of breeding males. Although there were matrix effects with blood and plasma, we can very tentatively suggest that 4OH-E2 (LLOQ 100 pg/mL), 2Me-E2 (LLOQ 2.5 pg/mL), and 4Me-E2 (LLOQ 1 pg/mL) were non-detectable in the blood and plasma of breeding males.

### 3.4 Discussion

In the present study, we developed a method to measure four estrogens (E1, 17β-E2, 17α-E2, and E3) with high specificity, sensitivity, accuracy, and precision. We also attempted to measure catechol and methoxy estrogens (2OH-E2, 4OH-E2, 2Me-E2 and 4Me-E2) but
encountered various problems. We employed DMIS, an estrogen-specific derivatization reagent, with LC-MS/MS. We validated DMIS derivatization for microdissected brain tissue (1-2 mg) whereas previous work applied DMIS only with serum samples. Assay sensitivity was improved by 10-fold for some estrogens and is among the best reported in the literature. We found substantial regional and seasonal variation in neuroestrogen levels in male song sparrows. For example, the NCM, a region with high aromatase expression, has the highest E1 and 17β-E2 levels. Estrogen levels in blood are very low. Lastly, estrogen levels are lower in the non-breeding season than in the breeding season.

3.4.1 Estrogen measurement

Estrogens are present at low concentrations and similar in structure (Figure 3.1), and therefore it is challenging to measure estrogens in biological samples. Historically, estrogens have been measured with immunoassays, but these can lack the necessary specificity due to antibody cross-reaction (Faupel-Badger et al., 2010; Haisenleder et al., 2011). LC-MS/MS has higher specificity than immunoassays (Grebe and Singh, 2011; Rosner et al., 2013; Gravitte et al., 2021) and can be combined with derivatization to measure various endogenous estrogens.

Several derivatization methods are used for estrogen measurement with LC-MS/MS. Dansyl chloride is the most widely used derivatization reagent for 17β-E2 measurement. However, the product ion is generated from the dansyl moiety and is not specific for the analyte by mass (Xu and Spink, 2008; Li and Franke, 2015). Moreover, dansyl chloride does not provide the sensitivity required for measurement of estrogens in microdissected brain tissue (C.J. and K.K.S., unpub. results). The reagent methyl-1-(5-fluoro-2,4-dinitrophenyl)-4-methylpiperazine (MPPZ) is useful for estrogen measurement but requires two reactions (Denver, Khan,
Stasinopoulos, Church, N. Z. Homer, et al., 2019). The reagent 2-fluoro-1-methylpyridinium-p-toluenesulfonate (FMP-TS) can be used to measure E\textsubscript{1} and 17β-E\textsubscript{2}, but the derivatives decline after only 2 days of storage at -20°C (Faqehi et al., 2016). Other reagents require complex sample preparation protocols, which can be time- and labor-intensive (Wudy et al., 2018; Denver, Khan, Homer, et al., 2019).

DMIS has several advantages in comparison to other derivatization reagents. First, the protocol is straightforward, consisting of a single reaction with relatively mild conditions. Second, DMIS derivatization provides high specificity because product ions are analyte-specific by mass. Third, assay sensitivity is among the best reported in the literature. Fourth, DMIS reacts specifically with estrogens and allows the simultaneous measurement of non-derivatized androgens and derivatized estrogens in the same sample (Keski-Rahkonen et al., 2015; Handelsman et al., 2020).

The present study is a step forward from the pioneering work by the Handelsman group. First, DMIS was used to quantify estrogens in human and mouse serum but not in brain (Keski-Rahkonen et al., 2015; Handelsman et al., 2020). In the present study, DMIS was used for the first time to measure brain estrogens. Second, we reduced reagent evaporation during the derivatization reaction. Third, the previous studies focused and 17β-E\textsubscript{2} and E\textsubscript{1}, and we added 17α-E\textsubscript{2} and E\textsubscript{3} to the panel. Fourth, we tested long-term stability of the derivatized analytes. Fifth, the previous studies used atmospheric pressure photoionization, which is relatively uncommon. This study used electrospray ionization, which is common, and makes the protocol more broadly applicable. Lastly, we tested stability of the deuterated IS and validated the use of 17β-E\textsubscript{2}-d\textsubscript{4} for DMIS derivatization (see below).
3.4.2 Method development

Deuterated IS are more widely available and affordable than 13C labeled IS. However, deuterated IS can be subject to hydrogen-deuterium exchange (Wudy et al., 2018). Here, we tested the stability of deuterated 17β-E2-d4 and did not observe deuterium loss (Figure 3.5). Furthermore, in several brain regions (e.g. NCM, POA, VTA), the 17β-E2 levels observed here are similar to those observed without DMIS (Jalabert, Ma and Soma, 2021) indicating that DMIS derivatization yields accurate levels of 17β-E2. In addition, the QCs showed high accuracy and precision for E₁, 17β-E₂, 17α-E₂, and E₃ (Table 3.3). Low accuracy and precision in QCs often indicate hydrogen-deuterium exchange. Lastly, the same deuterated IS was used previously for derivatization with DMIS and performed well, although the IS stability was not directly assessed in these studies (Keski-Rahkonen et al., 2015; Handelsman et al., 2020).

We assessed several assay parameters. Assay specificity is key because many estrogens are similar in structure (Figure 3.1). Here, E₁, 17β-E₂, 17α-E₂, E₃, 2Me-E₂, and 4Me-E₂ showed analyte-specific transitions patterns by mass and retention time (Table 3.1) whereas the 2OH-E₂ and 4OH-E₂ isomers were not distinguishable due retention time overlap. Assay sensitivity is also critical because estrogen amounts in blood and microdissected brain regions are extremely low. Here, DMIS derivatization improved the lower limit of quantification for all 7 estrogens (Table 3.2). The largest increases in sensitivity (10-fold) were observed for 17β-E₂, 2Me-E₂ and E₃. This allowed measurement of E₁ and 17β-E₂ in regions in which we previously could not (Jalabert, Ma and Soma, 2021). We also assessed assay accuracy and precision, which were acceptable in all cases (Table 3.3). Stability of all 7 derivatized estrogens was acceptable after storage in the autosampler (15°C) for 24 h, similar to previous results on 17β-E₂ (Keski-Rahkonen et al., 2015). Here, DMIS derivatives of E₁, 17β-E₂, 17α-E₂, and E₃ showed good
long-term stability (Table 3.5). However, derivatized catechol and methoxy estrogens were less stable, perhaps because of oxidation (Maclusky et al., 1981). Derivative stability is an important factor but often not reported (Denver, Khan, Homer, et al., 2019). No estrogens were measured in any blanks, and some biological samples (e.g. plasma samples from non-breeding sparrows) had non-detectable estrogen levels, indicating that this ultrasensitive assay does not produce “false positives.” Moreover, 17β-E2 levels in breeding NCM were very similar to previous results (without DMIS) (Jalabert, Ma and Soma, 2021). Overall, indices of assay performance were acceptable for E1, 17β-E2, 17α-E2, and E3.

Neuroestrogen measurement is also challenging due to the large amount of brain lipids that can interfere with assays (Taves et al., 2011). While many steroid extraction protocols are complex, ours is straightforward and rapid. Smaller tissue samples, as obtained by microdissection, contain less myelin and thus lower matrix effects. Estrogen measurement in large brain samples with dansyl chloride required a matrix surrogate for calibration curves, as matrix effects were present after extraction (Li and Gibbs, 2019). However, due to the limited amount of tissue obtained by microdissection (1-2mg), there is a trade-off between reducing matrix effects and obtaining detectable quantities of estrogens. We used several techniques to assess potential matrix effects. No matrix effects were detectable with brain tissue for E1, 17β-E2, 17α-E2, and E3. In contrast, matrix effects were present with brain tissue for 4OH-E2, 2Me-E2, and 4Me-E2 and suggest ion enhancement (Wudy et al., 2018). Similar results were observed in blood and plasma (Table 3.3 and Table 3.4).

Catechol and methoxy estrogens are challenging to measure (Maclusky et al., 1981; Mesaros, Wang and Blair, 2014) and we faced some difficulties for their quantification. We could not distinguish between 2OH-E2 and 4OH-E2 due to co-elution. A study using the
derivatization reagent MPPZ had the same issue (Denver, Khan, Stasinopoulos, Church, N. Z. M. Homer, et al., 2019) which was partially overcome by altering the liquid chromatography (Denver, Khan, Stasinopoulos, Church, N. Z. M. Homer, et al., 2019). Derivatization reagents can interact with either (or both) hydroxyl groups in the aromatic ring, which can hinder measurements (Denver, Khan, Stasinopoulos, Church, N. Z. Homer, et al., 2019). However, DMIS produced only double derivatives, which avoided this problem. The labile nature of catechol and methoxy estrogens is shown by our stability data (Table 3.5). Lastly, these analytes suffered from matrix effects (Table 3.3). Future studies can include additional IS for catechol and methoxy estrogens to correct for matrix effects. Overall, the current assay is sufficient to determine the presence or absence of 4OH-E2, 2Me-E2, and 4Me-E2 in brain, blood and plasma samples but not sufficient for quantification of these analytes.

3.4.3 Estrogen levels in song sparrow circulation

We examined estrogens in the circulation of wild male song sparrows. No estrogens were detectable in the circulation of non-breeding males. In the breeding season, we observed very low concentrations of blood E1 and 17β-E2 (detectable in 50% and 60% of samples, respectively) and of plasma E1 and 17β-E2 (detectable in 70% of samples). This is consistent with previous studies in song sparrows that showed a small increase in plasma 17β-E2 only at the beginning of the breeding season (Soma and Wingfield, 1999). Plasma 17β-E2 levels were lower than those in our previous study using radioimmunoassay (Heimovics et al., 2016), probably due to the higher specificity of LC-MS/MS. 17α-E2 and E3 were not detected in blood or plasma samples from breeding males. Our data also suggest that 4OH-E2, 2Me-E2, and 4Me-E2 are very low in the
circulation of male song sparrows. In addition, our data suggest that circulating levels of 2OH-E2 are very low, because we could not distinguish it from 4OH-E2.

3.4.4 Estrogen levels in song sparrow brain regions

Estrogens are locally synthesized within the songbird brain. They can be produced either de novo from cholesterol or from conversion of circulating precursors (Schmidt et al., 2008; Jalabert, Ma and Soma, 2021). Key steroidogenic enzymes, such as 3β-hydroxysteroid dehydrogenase/Δ5–Δ4 isomerase (3β-HSD) (London et al., 2006), cytochrome P450 17α-hydroxylase/17,20-lyase (CYP17A1) (London, Boulter and Schlinger, 2003) and aromatase (Saldanha et al., 2000; Saldanha, Remage-Healey and Schlinger, 2013), are expressed in the songbird brain. In the song sparrow brain, activities of 3β-HSD and aromatase are region-specific and show seasonal changes (Soma et al., 2003; Pradhan et al., 2010). Thus, estrogen levels can differ greatly across specific brain regions. Many studies use whole brain or macro-dissection to collect large regions (e.g., forebrain or cerebral cortex), which lack spatial specificity (Li and Gibbs, 2019). In contrast, we used microdissected brain regions (1-2 mg), which allows for a simple extraction method (Grebe and Singh, 2011) and provides much greater spatial resolution.

We detected E1 and 17β-E2 in nearly all brain regions in breeding males. Overall, the present E1 and 17β-E2 brain levels match our previous data using a LC-MS/MS assay without derivatization (Jalabert, Ma and Soma, 2021). Importantly, the higher sensitivity of the current assay allowed us to detect estrogens in brain regions where we could not before, such as 17β-E2 in the BnST, CG and Cb, and both 17β-E2 and E1 in the LS and VTA. In the NAc, none of the
Estrogens on our panel were detectable, probably because of its small size (only one punch for NAc) and low aromatase expression (Soma et al., 2003).

Estrogen measurement in the brain is challenging, as suggested by previous studies using immunoassays. Studies from the same lab have not been able to replicate results using an immunoassay for measurement of 17β-E₂ in zebra finch brain microdialysate, which might be due to changes in the commercial immunoassay (de Bournonville, McGrath and Remage-Healey, 2020). Further, when 17β-E₂ was measured in the same sample by both LC-MS/MS and immunoassay in quail brain microdialysate, LC-MS/MS detected far lower 17β-E₂ concentrations than the immunoassay (de Bournonville et al., 2021), suggesting antibody cross-reactivity. When a panel of 14 estrogens was analyzed in quail brain microdialysate by LC-MS/MS, 17β-E₂ represented less than 20% of total estrogens and 2OH-E₁ levels were high (de Bournonville et al., 2021).

Estrogens in the SBN regulate a variety of social behaviors. The POA had high levels of estrogens (Figure 3.7). Similarly, in male quail, estrogens are higher in the POA than in the circulation (Liere et al., 2019). Further, in the male quail POA, sexual interactions rapidly modulate aromatase activity (Cornil et al., 2005) and estrogen levels (de Bournonville et al., 2021). In wild male song sparrows, aromatase is expressed in the POA, and aromatase activity in the POA-diencephalon is higher in the breeding season than in the molt or non-breeding season (Soma et al., 2003). Local estrogen production in the POA likely promotes sexual behavior of male sparrows. The NCM is an auditory area that contains high levels of aromatase (Saldanha et al., 2000; Soma et al., 2003) and here showed the highest levels of E₁ and 17β-E₂ in breeding males. In zebra finches, 17β-E₂ levels in NCM rapidly increase in response to the presence of
females or when exposed to the song of another male (Remage-Healey, Maidment and Schlinger, 2008) suggesting a role for locally produced estrogens in social interactions.

There is dramatic seasonal variation in brain estrogen levels. In non-breeding males, we only detected $17\beta$-E$_2$ in the NCM. Improved sensitivity allowed the measurement of $17\beta$-E$_2$ in the NCM of non-breeding males, which was not possible without DMIS derivatization (Jalabert, Ma and Soma, 2021). Aromatase expression in the song sparrow brain is generally highest in the breeding season (Wacker et al., 2010). Consistent with this, the current data show that E$_1$ and $17\beta$-E$_2$ are more abundant and widespread in the brain during the breeding season. Nevertheless, neuroestrogens promote non-breeding aggression in male song sparrows (Soma, Tramontin and Wingfield, 2000; Heimovics, Ferris and Soma, 2015). Thus, neuro estrogen levels might be very low at baseline but increase rapidly in aggressive interactions, an idea that will be examined in a future study.

Here, we did not detect $17\alpha$-E$_2$, E$_3$, 4OH-E$_2$, 2Me-E$_2$, or 4Me-E$_2$ in any brain or blood samples from wild male song sparrows. The lack of $17\alpha$-E$_2$ in brain and circulation is consistent with the very low concentrations of $17\alpha$-testosterone (epitestosterone) in song sparrow plasma (Jalabert, Ma and Soma, 2021) as $17\alpha$-E$_2$ can be synthesized from $17\alpha$-testosterone (Finkelstein et al., 1981). $17\alpha$-E$_2$ is also absent in the brain and circulation of male quail (Liere et al., 2019). In quail, activity of estrogen-2-hydroxylase (CYP1A1; synthesizes 2OH-E$_2$ and 2OH-E$_1$) is elevated within the SBN (Balthazart et al., 1994). Catechol estrogens are then methylated by catechol O-methyl transferase (COMT) to produce methoxy estrogens. Here, non-detectable 4OH-E$_2$ suggests that 2OH-E$_2$ levels are also very low. The lack of 2Me-E$_2$ is consistent with data from quail brain. In this study, males had not been challenged (no simulated territorial
intrusion), which could explain why we did not detect catechol and methoxy estrogens. Future
work will examine the effects of a conspecific aggressive interaction.

3.5 Conclusions

In the present study, we developed a method to measure E₁, 17β-E₂, 17α-E₂, and E₃ in
brain, blood, and plasma. The derivatization improved sensitivity, making this assay among the
most sensitive reported in the literature. Further, the assay showed high specificity, accuracy, and
precision. Its application to the song sparrow model provides insights into the neural synthesis of
estrogens in songbirds. DMIS derivatization will have wide-ranging applications for measuring
estrogens in songbirds and other animal models as well as in humans.
### 3.6 Figures and tables

Table 3.1: Scheduled multiple reaction monitoring for the estrogens with 1,2-dimethylimidazole-5-sulfonylechloride (DMIS) and without derivatization

<table>
<thead>
<tr>
<th></th>
<th>Mode</th>
<th>Retention time (min)</th>
<th>Quantifier m/z</th>
<th>Qualifier m/z</th>
</tr>
</thead>
<tbody>
<tr>
<td>E₁-DMIS</td>
<td>ESI +</td>
<td>10.46</td>
<td>429 → 365.2</td>
<td>429 → 96.0</td>
</tr>
<tr>
<td>17β-E₂-DMIS</td>
<td>ESI +</td>
<td>10.74</td>
<td>431.0 → 367.4</td>
<td>431.0 → 95.9</td>
</tr>
<tr>
<td>17α-E₂-DMIS</td>
<td>ESI +</td>
<td>10.88</td>
<td>431 → 367</td>
<td>431 → 96.0</td>
</tr>
<tr>
<td>E₃-DMIS</td>
<td>ESI +</td>
<td>7.95</td>
<td>447 → 383.3</td>
<td>447 → 96.1</td>
</tr>
<tr>
<td>2OH-E₂-DMIS</td>
<td>ESI +</td>
<td>10.62</td>
<td>605.1 → 382.3</td>
<td>605.1 → 96.1</td>
</tr>
<tr>
<td>4OH-E₂-DMIS</td>
<td>ESI +</td>
<td>10.62</td>
<td>605.1 → 382.3</td>
<td>605.1 → 96.1</td>
</tr>
<tr>
<td>2Me-E₂-DMIS</td>
<td>ESI +</td>
<td>10.99</td>
<td>461 → 302</td>
<td>461 → 161.1</td>
</tr>
<tr>
<td>4Me-E₂-DMIS</td>
<td>ESI +</td>
<td>10.57</td>
<td>461 → 161.2</td>
<td>461 → 283.0</td>
</tr>
<tr>
<td>17β-E₂-d₄-DMIS</td>
<td>ESI +</td>
<td>10.73</td>
<td>435 → 371.1</td>
<td>435 → 96.1</td>
</tr>
<tr>
<td>E₁</td>
<td>ESI -</td>
<td>7.61</td>
<td>269 → 145</td>
<td>269 → 143.0</td>
</tr>
<tr>
<td>17β-E₂</td>
<td>ESI -</td>
<td>7.7</td>
<td>271 → 145</td>
<td>271 → 143.0</td>
</tr>
<tr>
<td>17α-E₂</td>
<td>ESI -</td>
<td>8.22</td>
<td>271 → 145</td>
<td>271 → 143.0</td>
</tr>
<tr>
<td>E₃</td>
<td>ESI -</td>
<td>3.56</td>
<td>287.1 → 171</td>
<td>287.1 → 144.9</td>
</tr>
<tr>
<td>2Me-E₂</td>
<td>ESI -</td>
<td>8.49</td>
<td>301.1 → 286</td>
<td>301.1 → 285.0</td>
</tr>
<tr>
<td>4Me-E₂</td>
<td>ESI -</td>
<td>8.1</td>
<td>301.1 → 286</td>
<td>301.1 → 285.0</td>
</tr>
<tr>
<td>17β-estradiol-d₄</td>
<td>ESI -</td>
<td>7.63</td>
<td>275 → 147</td>
<td>-</td>
</tr>
</tbody>
</table>
Table 3.2: Lower limit of quantification improved by DMIS

<table>
<thead>
<tr>
<th></th>
<th>Underivatized (pg/tube)</th>
<th>Derivatized (pg/tube)</th>
</tr>
</thead>
<tbody>
<tr>
<td>E₁</td>
<td>0.1</td>
<td>0.02</td>
</tr>
<tr>
<td>17β-E₂</td>
<td>0.2</td>
<td>0.02</td>
</tr>
<tr>
<td>17α-E₂</td>
<td>0.1</td>
<td>0.02</td>
</tr>
<tr>
<td>Estriol</td>
<td>0.2</td>
<td>0.02</td>
</tr>
<tr>
<td>4OH-E₂</td>
<td>-</td>
<td>2</td>
</tr>
<tr>
<td>2Me-E₂</td>
<td>0.5</td>
<td>0.05</td>
</tr>
<tr>
<td>4Me-E₂</td>
<td>0.5</td>
<td>0.2</td>
</tr>
</tbody>
</table>
Table 3.3: Accuracy, precision, and recovery in different matrices.

<table>
<thead>
<tr>
<th></th>
<th>Low QC (0.5 pg)</th>
<th>High QC (2 pg)</th>
<th>Brain (0.5-4 mg)</th>
<th>Blood (1-20 µL)</th>
<th>Plasma (1-20 µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>E₁</td>
<td>% Recovery (% CV)</td>
<td>93 (14)</td>
<td>97 (7)</td>
<td>102 (5)</td>
<td>90 (6)</td>
</tr>
<tr>
<td>17β-E₂</td>
<td>% Recovery (% CV)</td>
<td>97 (5)</td>
<td>101 (5)</td>
<td>102 (6)</td>
<td>94 (8)</td>
</tr>
<tr>
<td>17α-E₂</td>
<td>% Recovery (% CV)</td>
<td>89 (5)</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>E₃</td>
<td>% Recovery (% CV)</td>
<td>97 (6)</td>
<td>95 (5)</td>
<td>93 (3)</td>
<td>92 (6)</td>
</tr>
<tr>
<td>4OH-E₂</td>
<td>% Recovery (% CV)</td>
<td>107 (11)</td>
<td>78 (16)</td>
<td>613 (22)</td>
<td>n.d.</td>
</tr>
<tr>
<td>2Me-E₂</td>
<td>% Recovery (% CV)</td>
<td>96 (13)</td>
<td>104 (13)</td>
<td>214 (33)</td>
<td>295 (3)</td>
</tr>
<tr>
<td>4Me-E₂</td>
<td>% Recovery (% CV)</td>
<td>99 (8)</td>
<td>92 (10)</td>
<td>247 (14)</td>
<td>200 (5)</td>
</tr>
</tbody>
</table>

Note: Accuracy was measured by the recovery of a quality control with a known concentration of estrogen. Precision was measured by the coefficient of variation (CV) of replicates. Recovery was assessed for brain, blood, and plasma by comparing unspiked samples with samples spiked with a known amount of steroid. Recovery was not assessed for 17α-E₂ and only low QC was used for accuracy and precision so dashes are placed in those cells. n.d. is defined as non-detectable.
Table 3.4: Assay linearity in different matrices of song sparrow

<table>
<thead>
<tr>
<th></th>
<th>Brain</th>
<th>Blood</th>
<th>Plasma</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Δ slope (%)</td>
<td>Δ slope (%)</td>
<td>Δ slope (%)</td>
</tr>
<tr>
<td>E₁</td>
<td>7</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>17β-E₂</td>
<td>2</td>
<td>1</td>
<td>6</td>
</tr>
<tr>
<td>E₃</td>
<td>7</td>
<td>5</td>
<td>1</td>
</tr>
<tr>
<td>4OH-E₂</td>
<td>2</td>
<td>n.d.</td>
<td>14</td>
</tr>
<tr>
<td>2Me-E₂</td>
<td>1</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>4Me-E₂</td>
<td>19</td>
<td>3</td>
<td>2</td>
</tr>
</tbody>
</table>

Note: Difference in slope (Δ slope) was calculated by subtracting the slope of the sample with increasing amount with the slope of the standard curve in neat solution, and then dividing by the slope of the standard curve in neat solution multiplied by 100 and expressed in percentage (%). n.d. is defined as non-detectable.
Table 3.5: Stability of estrogen DMIS derivatives

<table>
<thead>
<tr>
<th></th>
<th>15°C</th>
<th>-20°C</th>
<th>-70°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Days</td>
<td>1</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>Estrone</td>
<td>100</td>
<td>99</td>
<td>94</td>
</tr>
<tr>
<td>17β-estradiol</td>
<td>102</td>
<td>101</td>
<td>99</td>
</tr>
<tr>
<td>17α-estradiol</td>
<td>122</td>
<td>132</td>
<td>115</td>
</tr>
<tr>
<td>Estriol</td>
<td>100</td>
<td>97</td>
<td>95</td>
</tr>
<tr>
<td>4OH-E₂</td>
<td>100</td>
<td>72</td>
<td>38</td>
</tr>
<tr>
<td>2Me-E₂</td>
<td>100</td>
<td>25</td>
<td>79</td>
</tr>
<tr>
<td>4Me-E₂</td>
<td>100</td>
<td>66</td>
<td>115</td>
</tr>
</tbody>
</table>

Note: stability was assessed in standard extracts following storage at different temperatures. Storage times were 1, 4, 8, and 31 days and temperatures were 15°C (autosampler), -20°C, and -70°C. Values were calculated as the percentage to the area ratio measured at time zero. Time zero was defined as the timepoint when injection into the LC-MS/MS occurs immediately following derivatization.
Figure 3.1: The panel of estrogens that are measured in this study.

Chemical structures of estrone, 17β-estradiol, 17α-estradiol, estriol, 2-hydroxyestradiol, 4-hydroxyestradiol, 2-methoxyestradiol, and 4-methoxyestradiol. Similarly, estrone can be hydroxylated at the 2 or 4 positions by CYP1A1 and CYP1B1 respectively, and then methylated by the COMT enzyme to produce 2-methoxyestrone and 4-methoxyestrone. Figure contributions: illustration created by M. Salehzadeh.
Figure 3.2: Representation of punch locations in brain slices showed in a rostral to caudal order

Punches were 1mm diameter and are shown to scale. Diagrams were adapted from the zebra finch atlas (Nixdorf-Bergweiler & Bischof, 2007). Abbreviations: NAc, nucleus accumbens; POA, preoptic area; AH, anterior hypothalamus; LS, lateral septum; BnST, bed nucleus of the stria terminalis; VMH, ventromedial hypothalamus; VTA, ventral tegmental area; CG, central grey; NCM, caudomedial nidopallium; TnA, nucleus taeniae of the amygdala; Cb, cerebellum. Adapted from Jalabert et al., 2021. Figure contributions: illustration created by M. Salehzadeh.
Figure 3.3: Derivatization reaction with 1,2-dimethylimidazole-5-sulfonyl-chloride (DMIS)

All estrogens of the panel react with DMIS similarly except the catechol estrogens where DMIS reacts with both hydroxyl groups of the aromatic ring. Figure contributions: illustration created by M. Salehzadeh.
Figure 3.4: Representative chromatograms for 1 pg of each estrogen (A) without and (B) with derivatization

Intensity is expressed in cps, time is expressed in minutes.
Figure 3.5: The mass spectra of Q1 scan (left panel) and the multiple reaction monitoring (right panel) of 17β-estradiol directly from the stock solution (A, B) or after derivatization (C, D) and 17β-estradiol-2,4,16,16-d4 directly from the stock solution (E, F), after sham derivatization (G, H), or after derivatization (I, J).

Abbreviations: quadrupole 1 (Q1); quadrupole 3 (Q3); m/z, mass-to-charge ratio.
Figure 3.6: calibration curves ranging from 0.02 to 20 pg with insets displaying the lowest standards on the curve for (A) estrone, (B) 17β-estradiol, (C) 17α-estradiol, and (D) estriol.

Area ratio is calculated by dividing an analyte peak area with the internal standard peak area in the same sample.
Figure 3.7: Male breeding song sparrows brain and blood levels of (A) estrone and (B) 17β-estradiol. Bar graphs represent the concentration of estrogens (ng/g brain tissue and ng/mL for blood).

Values are expressed as the mean ± standard error of mean (SEM), n = 10. Abbreviations from left to right: WB, blood; NAc, nucleus accumbens; POA, preoptic area; AH, anterior hypothalamus; LS, lateral septum; BnST, bed nucleus of the stria terminalis; VMH, ventromedial hypothalamus; VTA, ventral tegmental area; CG, central grey; NCM, caudomedial nidopallium; TnA, nucleus taeniae of the amygdala; Cb, cerebellum.
4.1 Introduction

Aggression is one of the most important social behaviors and plays key roles in many life history stages. For example, in the breeding season, males compete for access to breeding females, and competitors evaluate strength and establish dominance in front of a potential mate. Females are also aggressive towards conspecifics when defending their offspring or when competing for access to limited nesting sites. Beyond reproductive contexts, animals compete for acquisition of territories that provide food and shelter, which are crucial during the non-breeding season. Consequently, aggressive interactions have a direct impact on the reproductive success and survival of individuals (Wingfield et al., 2006).

Social behaviors, including aggression, are regulated by a neural circuit known as the social decision-making network (SDMN), which includes the social behavior network and the mesocorticolimbic reward system (O’Connell and Hofmann, 2011). These brain areas are located in the forebrain, midbrain, and hindbrain, and the circuit regulates multiple forms of social behavior, including different types of aggression. Further, every node of the SDMN expresses sex steroid receptors, indicating that these brain regions are sex steroid-sensitive and that hormones play key roles in modulating the activity of this network and in regulating social behaviors.

In seasonally-breeding vertebrates, the gonads grow before the breeding season and regress following the termination of breeding. Circulating sex steroid concentrations fluctuate
alongside gonadal growth and regression, and high levels of aggression generally occur when circulating levels of sex steroids are high in reproductive contexts (Wingfield et al., 1990). Castration and replacement experiments have shown that breeding aggression depends on gonadal testosterone (T) (Vandenbergh, 1971; Balthazart, 1983; Moore and Marler, 1987; Wingfield and Hahn, 1994). Interestingly, during the non-breeding season, aggression persists in many species, despite low circulating sex steroid levels and even after castration. Non-breeding aggression might depend on steroids from a non-gonadal source (Soma et al., 2008).

The brain itself can locally produce steroids, either de novo from cholesterol or from conversion of circulating precursors (Schmidt et al., 2008; Schlinger, 2015). This is possible as the brain expresses all the necessary enzymes for steroid synthesis (Tsutsui, 2011; Hojo and Kawato, 2018). The steroidogenic enzymes show a seasonal pattern and are expressed in a brain region-specific manner that correlates with seasonal changes in aggression (Soma et al., 2003). Brain-derived steroids, known as neurosteroids, were first characterized in rodents (Baulieu, 1991; Mellon, Griffin and Compagnone, 2001; Hojo and Kawato, 2018) and later in other vertebrates such as birds (Schmidt et al., 2008; Tsutsui, 2011; Schlinger, 2015).

Song sparrows, *Melospiza melodia*, are territorial year-round and therefore are an excellent animal model to study seasonal changes in the steroid modulation of aggression (Wingfield and Soma, 2002). Breeding males are highly territorial, and this behavior is supported by gonadal T, and androgen receptor antagonism reduces aggression in the breeding season (Sperry, Wacker and Wingfield, 2010). Interestingly, non-breeding males also defend territories aggressively, although the testes are regressed, systemic T levels are very low, and castrations does not reduce aggression (Wingfield, 1994). However, sex steroids still play regulate non-breeding aggression, as aromatase inhibition (but not androgen receptor antagonism) decreases
aggressive behavior (Soma et al., 2000; Soma, Tramontin and Wingfield, 2000; Sperry, Wacker and Wingfield, 2010).

In song sparrows, steroidogenic enzyme activity in the brain changes with season and social context. An enzyme necessary to produce active androgens, 3β-hydroxysteroid dehydrogenase/Δ5–Δ4 isomerase (3β-HSD), is higher in the diencephalon and telencephalon during the non-breeding season than the breeding season, and 3β-HSD activity in the forebrain is rapidly upregulated by an aggressive interaction in the non-breeding season (Pradhan et al., 2010). Activity of 5α-reductase, which converts T to 5α-dihydrotestosterone (5α-DHT), is high in diencephalic and telencephalic regions during the breeding season (Soma et al., 2003). Aromatase, which converts androgens into estrogens, is expressed during both breeding and non-breeding seasons (Soma et al., 2003; Wacker et al., 2010), and aromatase inhibition decreases non-breeding aggression (Soma et al., 2000; Soma, Tramontin and Wingfield, 2000). Together, these data provide evidence of neural steroidogenesis and suggest that some enzymes are upregulated during the non-breeding season.

Here, we studied the acute effects of a 10-min simulated territorial intrusion (STI) on behavior and steroid levels during the breeding and non-breeding seasons in free-living male song sparrows. We examined steroids in whole blood, plasma and 11 brain regions. We measured progesterone, corticosterone, T, and 5α-DHT. We evaluated rapid changes in response to aggression to further understand how complex social interactions, such as aggressive behavior, rapidly might affect local steroid synthesis in the brain.
4.2 Materials and methods

4.2.1 Field procedures

Free-living adult male song sparrows in breeding (April 25th to May 8th, 2019) and non-breeding seasons (October 16th to November 3rd, 2019) were used. Field sites were located near Vancouver, British Columbia, Canada. In both seasons, subjects were randomly assigned to one of two treatment groups: simulated territorial intrusion (STI) or control (CON) (n=10 per group in breeding season, n=9 per group in non-breeding season). STI animals were exposed to conspecific song playback from a speaker and a live caged conspecific male decoy placed in their territories for 10 min (duration was determined in a pilot study). The playback song included songs from several individuals, with 10 repetitions per song at 10 sec intervals (Wingfield, 1985; Maddison et al., 2012). For CON animals, an empty cage and a silent speaker were placed in the territories for 10 min. During the STI or CON condition, we measured aggressive responses as before (according to (Wingfield, 1985; Wingfield and Hahn, 1994)), including: (i) response latency, (ii) number of songs, (iii) number of flights, and (iv) time spent within 5m of the decoy. Response latency is defined as the time to first response of any kind. After 10 min of STI or CON, a mist net (that was set up ahead of time) was rapidly unfurled and subjects were captured using a mist net and conspecific song playback for a maximum of 5 min (breeding STI: 1.5 ± 0.5 min, breeding CON: 2.1 ± 0.6 min, non-breeding STI: 1.8 ± 0.8 min, non-breeding CON: 1.9 ± 0.6 min). The amount of playback used to catch subjects was similar in all 4 groups (F3,34 = 0.714, p = 0.5502). Immediately after capture, the subject was rapidly and deeply anesthetized with isoflurane and then rapidly decapitated. It was a maximum of 3 min between catching the subject to euthanasia, to avoid effects of handling on steroid levels (breeding STI: 2.5 ± 0.2 min, breeding CON: 2.5 ± 0.1 min, non-breeding STI: 2.6 ± 0.1 min,
non-breeding CON: $2.8 \pm 0.05$ min). Handling duration was similar in all 4 groups ($F_{3,34} = 1.37$, $p = 0.2685$). The brain was immediately dissected from the skull and snap frozen on powdered dry ice. Trunk whole blood was collected in heparinized microhematocrit tubes (Fisher Scientific) that were kept on ice packs until returned to the laboratory (within 5h). Once in the laboratory, whole blood was divided into 2 aliquots; one half of the blood sample was frozen and the other half was centrifuged and plasma was collected. All samples were stored at $–70^\circ$C until steroids were extracted.

Circulating levels of steroids were measured in both whole blood (5 μL per sample) and plasma (10 μL per sample). We used blood to assess circulating steroid levels and to compare with brain steroid levels, because the use of plasma overestimates steroid concentrations in the circulation (Taves et al., 2010, 2011, 2015). We used plasma to match previous reports in song sparrows.

All procedures were in compliance with the Canadian Council on Animal Care and protocols were approved by the Canadian Wildlife Service and the UBC Animal Care Committee.

### 4.2.2 Brain microdissection

The Palkovits punch technique (Palkovits, 1973) was used as before (Charlier et al., 2011; Heimovic et al., 2016; Jalabert, Ma and Soma, 2021). Microdissected brain tissue was collected from 11 brain areas SDMN (O’Connell and Hofmann, 2011): nucleus accumbens (NAc), preoptic area (POA), anterior hypothalamus (AH), lateral septum (LS), bed nucleus of the stria terminalis (BnST), ventromedial hypothalamus (VMH), ventral tegmental area (VTA), central grey (CG), caudomedial nidopallium (NCM), nucleus taeniae of the amygdala (TnA).
(homolog of the mammalian medial amygdala), and cerebellum (Cb). Brains were sectioned in the coronal plane at 300 μm on a MicroHM525 cryostat at –12°C (Thermo Fisher Scientific Inc., Waltham, MA) using a plane of sectioning that closely matched a zebra finch brain atlas (Nixdorf-Bergweiler and Bischof, 2007) as before (Heimovics et al., 2016; Jalabert, Ma and Soma, 2021). Sections were mounted onto Superfrost Plus microscope slides (Thermo Fisher Scientific Inc.) and kept cold on dry ice during punching. The specific brain regions of interest listed above were microdissected using an Integra Miltex stainless-steel biopsy punch tool (1 mm diameter, tissue wet weight 0.245 mg per punch (Taves et al., 2011), Fisher Scientific). The same punch size was used for all brain areas. Depending on the size of the brain area, a total of 1, 4 or 6 punches were collected and tissue amount was 0.245, 0.98, or 1.47mg, respectively.

Brain microdissection was performed as before (Heimovics et al., 2016; Jalabert, Ma and Soma, 2021). Specifically, one punch (centered at the midline for one section) was collected containing the NAc, ventral to the Area X. Four punches (two per side for two serial sections) containing the POA were collected immediately caudal to the last section containing the tractus septopalliomesencephalicus (TrSM). Four punches (two per side for two serial sections) containing the AH were collected immediately caudal to the POA sections and ventral to the anterior commissure (CoA). Four punches (two per side for two serial sections) containing the LS and BnST were collected dorsal to the CoA, the LS medial to the lateral ventricles and the BnST at the tip of each lateral ventricle. Four punches (two per side for two serial sections) containing the VMH were collected ventral to the AH. Four punches (two per side for two serial sections) containing the VTA were collected from two serial sections ventrolateral to the oculomotor nerve NIII. Four punches (two per side for two serial sections) containing the CG were collected from two serial sections ventral to the posterior commissure. Six punches (two
per side for three serial sections) containing the NCM were collected starting at the last appearance of the CoA and tractus occipito-mesencephalicus path from the ventromedial telencephalon. NCM punches did not include the song control nucleus HVC. Six punches (two per side for three serial sections) containing the TnA were collected immediately caudal to the disappearance of the CoA and tractus occipito-mesencephalicus. Six punches (two at the midline for three serial sections) containing the Cb were collected from its first appearance. Punches were expelled into 2-mL polypropylene tubes (Sarstedt AG & Co, Numbrecht Germany, 72.694.007) each containing five zirconium ceramic oxide beads (1.4-mm diameter) and stored at –70°C until processing.

4.2.3 Reagents

High Performance Liquid Chromatography (HPLC)-grade acetonitrile, hexane, and methanol were from Fisher Chemical. Certified reference standards were obtained from Cerilliant (Round Rock, TX). Stock solutions of steroids were prepared in HPLC-grade methanol and calibration curves were prepared in 50% methanol. The calibration curve ranged from 0.05 to 1000 pg/tube for corticosterone, progesterone, T, and 5α-DHT. Deuterated internal standards (IS) of progesterone-d9, corticosterone-d8, and testosterone-d5 (C/D/N Isotopes Inc., Pointe-Claire, Canada) stock solutions were prepared in methanol and further diluted with 50% methanol to a final working solution of 0.04 ng/mL for progesterone-d9 and testosterone-d5 and 0.4 ng/mL for corticosterone-d8.
4.2.4 Steroid extraction

Steroids were extracted from blood (5 µL), plasma (10 µL), and brain tissue (amount detailed above) using liquid-liquid extraction, as before (Tobiansky et al., 2020; Jalabert, Ma and Soma, 2021). Briefly, five zirconium ceramic oxide beads (1.4-mm diameter, Fisher Scientific) were added to each vial. One mL of acetonitrile was added to all samples, and 50 µL of the deuterated IS was added to all samples except “double blanks” to track recovery and matrix interference for each sample. Then, samples were homogenized using a bead mill homogenizer (Omni International Inc., Kennesaw, GA) at 4 m/s for 30 s. Then samples were centrifuged at 16,100g for 5 min, and 1 mL of supernatant was transferred to a borosilicate glass culture tube (12x75mm) (VWR International) pre-cleaned with methanol. Then 0.5 mL of hexane was added, and samples were vortexed and centrifuged at 3200g for 2 min. The hexane was removed and discarded, and the extracts were dried at 60°C for 45 min in a vacuum centrifuge (ThermoElectron SPD111V; Thermo Fisher Scientific). Samples were reconstituted with 55 µL of 25% methanol, transferred to 0.6 mL polypropylene microcentrifuge tubes (Fisher Scientific), and centrifuged at 16,100g for 2min. Then 50 µL of supernatant were transferred to a LC vial insert (Agilent, Santa Clara, CA, USA) and stored at -20°C until injection.

Samples were processed along with calibration curves, quality controls (QCs), blanks, and double blanks.

4.2.5 Steroid analysis by LC-MS/MS

Steroids were quantified using a Sciex QTRAP 6500 UHPLC-MS/MS system as previously described (Jalabert, Ma and Soma, 2021). Samples were transferred into a refrigerated autoinjector (15°C). Then, 45 µL of each sample were injected into a Nexera X2 UHPLC system
(Shimadzu Corp., Kyoto, Japan), passed through a KrudKatcher ULTRA HPLC In-Line Filter (Phenomenex, Torrance, CA, USA) followed by an Agilent 120 HPH C18 guard column (2.1 mm) and separated on an Agilent 120 HPH C18 column (2.1 x 50 mm; 2.7 μm; at 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. During loading, MPB was at 10% for 0.5 min, from 0.6 to 4 min the gradient profile was at 42% MPB, which was ramped to 60% MPB until 9.4 min. From 9.4 to 9.5 min the gradient was 60-70% MPB, which was ramped to 98% MPB until 11.9 min and finally a column wash from 11.9 to 13.4 min at 98% MPB. The MPB was then returned to starting conditions of 10% MPB for 1 min. Total run time was 14.9 min. The needle was rinsed externally before and after each sample injection with 100% isopropanol.

We used 2 multiple reaction monitoring (MRM) transitions for each steroid and 1 MRM transition for each deuterated internal standard (Jalabert, Ma and Soma, 2021). Steroid concentrations were acquired on a Sciex 6500 Qtrap triple quadrupole tandem mass spectrometer (Sciex LLC, Framingham, MA) in positive electrospray ionization mode for all four steroids. All water blanks were below the lowest standard on the calibration curves.

4.2.6 Statistical analysis

A value was considered non-detectable if it was below the lowest standard on the calibration curve. When 20% or more of the samples in a group (blood or brain region) were detectable, then the missing values were estimated via quantile regression imputation of left-censored missing data using MetImp web tool (Wei, Wang, Jia, et al., 2018; Wei, Wang, Su, et al., 2018; Tobiansky et al., 2020, 2021). Data were imputed for each season and behavioral
treatment independently. When less than 20% of the samples in a group (blood or brain region) were detectable, then imputations were not performed, data were not analyzed statistically, and data are only reported in the text. To compare steroid levels in brain and blood, we assumed that 1 mL of blood weighs 1 g (Taves et al., 2011; Tobiansky et al., 2020).

Statistics were conducted using GraphPad Prism version 9.02 (GraphPad Software, La Jolla, CA, USA). When necessary, data were log transformed prior to analysis. The effects of STI on steroid levels in the circulation were tested using a t-test and in the brain were analyzed using repeated measures two-way ANOVA with one between-subjects factor (STI) and one within-subjects factor (brain area). Analyses were followed by Sidak multiple comparison tests and corrected p values are shown. Significance criterion was set at p ≤ 0.05. Graphs show the mean ± standard error of the mean (SEM) and are presented using the non-transformed data.

4.3 Results

4.3.1 Aggressive behavior in the breeding and non-breeding seasons

The STI increased aggressive behavior in both breeding and non-breeding seasons (Figure 4.1 and Figure 4.2). For response latency, song latency, and flight latency, there was a significant main effect of STI but no significant main effect of season and no significant STI × season interaction. The STI decreased the response latency (F1,34 = 177.9, p < 0.0001; Figure 4.1 A), song latency (F1,34 = 125.7, p < 0.0001; Figure 4.1 B), and flight latency (F1,34 = 271.2, p < 0.0001; Figure 4.1 C).

For time in 5 m, there was a significant main effect of STI (F1,34 = 319.9, p < 0.0001; Figure 4.2 A), a significant main effect of season (F1,34 = 6.121, p = 0.0185) and a significant STI
x season interaction (F\(_{1,34} = 6.121\), p = 0.0185). Interestingly, post-hoc comparisons revealed that while STI treatment increased the time spent in 5 m in both seasons, STI subjects spent more time in 5 m during the non-breeding season than the breeding season (p = 0.0069; Figure 4.2 A).

For number of songs, there was a significant main effect of STI (F\(_{1,34} = 141.7\), p < 0.0001; Figure 4.2 B), a significant main effect of season (F\(_{1,34} = 4.374\), p = 0.044) but no significant interaction (F\(_{1,34} = 2.842\), p = 0.101).

For number of flights, there was a significant main effect of STI (F\(_{1,34} = 503.8\), p < 0.0001; Figure 4.2 C), a significant main effect of season (F\(_{1,34} = 5.755\), p = 0.0221) and a significant STI x season interaction (F\(_{1,34} = 10.35\), p = 0.0028). Post-hoc comparisons revealed that STI increased the number of flights in both seasons and that STI subjects made more flights during the non-breeding season than the breeding season (p = 0.0019; Figure 4.2 C).

### 4.3.2 Steroid levels in the circulation and brain of breeding males

We measured steroid levels changes in response to an aggressive interaction in the circulation and several microdissected brain regions of breeding subjects.

Progesterone was detectable in blood and most brain regions, and overall progesterone levels were higher in blood than in brain (Figure 4.3 A). STI did not affect progesterone levels in the blood (p = 0.1558). In the brain, progesterone did not show a significant main effect of STI (F\(_{1,18} = 0.0337\), p = 0.8564) but did show a significant main effect of region (F\(_{10,180} = 2.694\), p = 0.0042) and a significant STI x region interaction (F\(_{10,180} = 4.486\), p < 0.0001). Post-hoc comparisons revealed that STI decreased progesterone levels the NAc (p = 0.0004) whereas all the other brain areas remained unchanged (all p > 0.1).
Corticosterone levels were generally greater in blood than in brain (Figure 4.3 B). STI significantly increased corticosterone levels in the blood ($p = 0.0001$). In the brain, corticosterone showed a significant main effect of STI ($F_{1,18} = 11.34$, $p = 0.0034$), a significant main effect of region ($F_{10,180} = 34.39$, $p < 0.0001$) and a significant STI x region interaction ($F_{10,180} = 3.225$, $p = 0.0008$). Post-hoc comparisons revealed that STI significantly increased corticosterone levels in the POA, AH, LS, VMH, VTA, CG, and Cb (all $p < 0.05$), a trend to increase in the BnST ($p = 0.0558$) and NCM ($p = 0.10$), and no effect in the NAc and TnA (both $p > 0.1$).

For T, blood levels were generally higher than brain levels (Figure 4.3 C). In the blood, STI had no significant effect on T levels ($p = 0.5663$). In the brain, there was a significant main effect of region ($F_{10,180} = 68.45$, $p < 0.0001$) but no significant main effect of STI ($F_{1,18} = 0.0107$, $p = 0.9188$) nor an STI x region interaction ($F_{10,180} = 0.602$, $p = 0.8105$).

For 5α-DHT, blood levels were generally lower brain levels (Figure 4.3 D). In the blood, STI had no significant effect on 5α-DHT levels ($p = 0.1532$). In the brain, there was a significant main effect of region ($F_{10,180} = 37.58$, $p < 0.0001$) but no significant main effect of STI ($F_{1,18} = 0.667$, $p = 0.4248$) nor an STI x region interaction ($F_{10,180} = 0.533$, $p = 0.8649$).

### 4.3.3 Steroid levels in the circulation and brain of non-breeding males

We also examined the effect of STI in the blood and several microdissected brain regions in non-breeding subjects.

For progesterone, overall blood levels were higher than brain levels (Figure 4.4 A). STI significantly increased the levels of progesterone in blood ($p=0.0036$), AH ($p=0.0293$), VTA ($p=0.0254$), NCM ($p < 0.0001$), TnA ($p < 0.0001$), and Cb ($p=0.023$). In contrast STI decreased
progesterone levels in the POA (p = 0.0372) and did not affect progesterone levels in LS, BnST, VMH, and CG (all p > 0.1) and remained non-detectable in the NAc for both groups.

Corticosterone levels were generally greater in blood than in brain (Figure 4.4 B). STI significantly increased corticosterone levels in the blood (p < 0.0001). In the brain, corticosterone showed a significant main effect of STI (F_{1,16} = 30.91, p < 0.0001) and a significant main effect of region (F_{10,160} = 42.84, p < 0.0001) but not an STI x region interaction (F_{10,160} = 1.586, p = 0.1150).

T was non-detectable in the circulation of both CON and STI subjects (Figure 4.4 C). In contrast, STI increased T levels in POA (p < 0.0001), AH (p < 0.0001), and TnA (p < 0.0001). Interestingly, STI decreased T levels in NAc (p < 0.0001) and showed a trend to decrease in the LS (p = 0.062) whereas it remained unchanged in BnST, NCM, CG, and Cb (all p > 0.1) and VMH and VTA were undetectable in both CON and STI.

5α-DHT was non-detectable in all non-breeding samples.

4.4 Discussion

Here, we demonstrate precise regulation of local steroid levels in the brain of both breeding and non-breeding wild song sparrows. Corticosterone was rapidly modulated in brain and circulation during both the breeding and non-breeding seasons. In the non-breeding season, aggressive interactions induced rapid changes in T concentrations only in specific brain regions, while T was not detectable in circulation. Further, the effects of a brief social interaction on progesterone levels in non-breeding animals differed between the blood and the brain. Progesterone increased in the blood of STI animals, while there was a region-specific response in the brain. Specifically, we observed either upregulation, downregulation, or no change in neural
progesterone depending on the brain region. In contrast, during the breeding season, progesterone, T, and 5α-DHT levels were not modulated by STI. Notably, in all regions examined, neural 5α-DHT levels far exceeded circulating levels in breeding subjects. These data, together with previous results, suggest that neurosteroids are rapidly synthesized in the brain when circulating sex steroids are low. When circulating sex steroids are high, the time-frame for altering steroid levels might be longer. Song sparrows are thus, an excellent model to study neurosteroidogenesis and the seasonal switch between neuroendocrine mechanisms modulating social behavior that would avoid the costs of elevated systemic levels of steroids.

4.4.1 Seasonality of aggression

Wild male song sparrows displayed robust aggressive behavior during the STI in both breeding and non-breeding seasons. STI subjects showed similar behavioral latencies (response, song, and flight) in both seasons, as observed before (Newman and Soma, 2011). However, some particular aspects of behavior in response to STI differed between seasons. Time spent within 5 m of the decoy intruder and the number of flights were higher in the non-breeding season, whereas the number of songs was higher in the breeding season. This is different from previous reports in this species that did not find seasonal differences in these parameters (Wingfield and Hahn, 1994). One seasonal difference previously observed is the persistence of aggression after the termination of the STI (once the decoy and playback are removed) (Wingfield, 1994; Heimovics, Fokidis and Soma, 2013). In the current study, this aspect was not measured because animals were captured immediately after the termination of STI for steroid measurement. Control subjects only sang during the breeding season, as seen before (Newman and Soma, 2011). Spontaneous singing (without an STI) is more frequent during the breeding season, which
could serve reproductive purposes (Searcy, 1984; O’Loghlen and Beecher, 1999). Overall, both breeding and non-breeding males are highly aggressive as seen in previous reports (Wingfield and Soma, 2002).

4.4.2 Effects of STI on steroid levels in breeding males

Generally, in control subjects, levels of progesterone, corticosterone, T, and 5α-DHT observed in the present study are similar to the levels in our previous study using the same LC-MS/MS method (Jalabert, Ma and Soma, 2021). In the breeding season, progesterone, corticosterone, and T levels are lower in brain than in blood, whereas 5α-DHT levels are higher in brain than in blood. In birds, some steroids are transported in the blood by corticosteroid binding globulin (CBG), which binds glucocorticoids and progestogens with high affinity (Charlier et al., 2009; Vashchenko et al., 2016). However, no sex hormone binding globulin has been found in birds (Charlier et al., 2009; Vashchenko et al., 2016). Recent work has shown that, in birds, androgens can also be transported in circulation by CBG, though with less binding affinity than the other steroids (Vashchenko et al., 2016; Lin et al., 2021). Circulating steroids can only cross the blood-brain barrier when they are not bound to carrier proteins like CBG (Mendel, 1989; Hammond, 1995; Breuner and Orchinik, 2002). This suggests that there is even greater regulation of local steroid levels in the brain since only the free fraction of circulating steroids can access the brain. Here, all four steroids were detected in all 11 brain areas and, interestingly, local levels varied across brain regions showing similar patterns to those observed before (Jalabert, Ma and Soma, 2021). The male song sparrow brain expresses the steroidogenic enzymes necessary for the local regulation of these steroids. In the breeding season, 3β-HSD (necessary for both progesterone and T production) is expressed in the telencephalon (contains
NAc, POA, LS, BnST, NCM, and TnA), the diencephalon (contains AH and VMH), and the cerebellum. As for $5\alpha$-DHT synthesis, $5\alpha$-reductase is highly expressed in the breeding telencephalon, NCM, and diencephalon (Soma et al., 2003).

There was no effect of STI on progesterone levels in the breeding season. The relationship between progesterone and breeding aggression has been studied mostly in females, where a negative relationship is observed. In mammals, circulating progesterone levels decrease after an aggressive interaction in female California mice (Davis and Marler, 2003) and progesterone administration inhibits aggression in female Syrian hamsters (Kohlert and Meisel, 2001) and female rats (Albert, Jonik and Walsh, 1992). Similarly, in birds, circulating progesterone levels decrease after an aggressive interaction, and progesterone administration reduces aggression in female black coucals (Goymann et al., 2008). In contrast, in males, the results are mixed. Progesterone administration promotes aggression in male lizards (Weiss and Moore, 2004). An aggressive interaction increases plasma progesterone levels in male ovenbirds (Adreani, Goymann and Mentesana, 2018) but decreases plasma progesterone levels in male white-crowned sparrows (Charlier et al., 2009). However, these studies focus on systemic progesterone levels. This is the first study to also examine aggression and local progesterone levels. Here, the lack of STI effect on progesterone levels in breeding subjects might be related to the duration of the aggressive challenge (10 min), and future studies should examine longer time points (e.g. 30 min).

STI significantly increased corticosterone in both brain and blood during the breeding season. Similarly, an STI increases corticosterone levels in plasma from the brachial vein (indicating systemic steroid levels) and jugular vein (indirectly indicating neural steroid levels) (Newman and Soma, 2011) and aggression is positively correlated with circulating
corticosterone (Newman and Soma, 2011; Davies, Beck and Sewall, 2018). An aggressive challenge also increases plasma corticosterone in male white-crowned sparrows (Charlier et al., 2009) as well as the year-round territorial European nuthatch (Landys et al., 2010). Corticosterone mobilizes glucose and might be useful to meet the energetic demands of the behavioral response (Romero, 2002). Glucocorticoids have differential effects on aggression. Acutely, glucocorticoids promote aggression, whereas chronically, glucocorticoids decrease aggression. In rodents, inhibition of acute glucocorticoid synthesis decreases aggression (Mikics, Kruk and Haller, 2004; Fish, DeBold and Miczek, 2005) and acute glucocorticoid administration rapidly (within 10 min) increases aggression (Hayden-Hixson and Ferris, 1991; Mikics, Kruk and Haller, 2004). In contrast, prolonged corticosterone elevation inhibits aggression (Tokarz, 1987; DeNardo and Licht, 1993). In song sparrows, a chronic administration of corticosterone suppresses breeding aggression (Wingfield and Silverin, 1986).

During the breeding season, neither T nor 5α-DHT levels in blood and brain were rapidly affected by STI. Similarly, in free-living male song sparrows, a rapid (<10 min) aggressive interaction does not increase plasma T levels (Wingfield and Wada, 1989). However, an increase in circulating T occurs when males are challenged for longer periods of time (e.g. 30 min) (Wingfield, 1985; Wingfield and Wada, 1989). In a laboratory study, a 5-min aggressive interaction also does not affect T levels (Heimovics et al., 2016). Likewise, there was no effect of social interaction on circulating 5α-DHT in the current study, which is consistent with previous results (Wingfield, 1985). No changes to 5α-DHT in the brain were observed. However, the importance of circulating androgens in promoting aggression in breeding male song sparrows is well established (Wingfield, 1984, 1985, 1994; Wingfield and Hahn, 1994; Sperry, Wacker and Wingfield, 2010). Importantly, the timing of these studies is from several hours and even
weeks, and it is proposed that an increase in circulating androgens is related to the expression of aggression in subsequent social interactions the maintenance of a dominant status (Oliveira, Silva and Canário, 2009).

4.4.3 Effects of STI on steroid levels in non-breeding males

Here, we found that male non-breeding song sparrows have a precise local steroid regulation in the brain and a social aggressive interaction induces region-specific changes in concentrations of progesterone, glucocorticoids, and androgens. Interestingly, compared to blood, levels of progesterone and corticosterone were lower in the brain whereas T showed the opposite pattern. All three steroids showed a main effect of region which further indicates that there is region-specific regulation. Although progesterone increased in blood of STI animals, its local regulation in the brain seems to be more precise as it increased in AH, VTA, NCM, TnA, and Cb but decreased in the POA and remained unchanged in other regions. Importantly, T was non-detectable in the blood of either CON or STI animals, but showed rapid changes in the brain. These results suggest the fine tuning of neural steroid levels in the non-breeding season.

Neural progesterone can promote non-breeding aggression by acting directly on the brain or acting indirectly after local conversion to other steroids. Progesterone receptors (PR) are present in specific regions of the avian brain (Lea, Clark and Tsutsui, 2001; Camacho-Arroyo et al., 2007). Here, STI increased progesterone levels in the AH but decreased levels in the POA and had no effect in the VMH. In male mice, PR-expressing neurons in the VMH promote aggression, independent of the gonads (Yang et al., 2017). Interestingly, these neurons project to POA and periaqueductal gray (homolog of the avian CG) (Yang et al., 2013). In male non-breeding Siberian hamsters, aggression is positively correlated with progesterone levels in the
LS, AH and medial amygdala (homolog of the avian TnA) (Munley et al., 2021). Here, progesterone levels in AH and TnA were upregulated by the STI. Progesterone could serve as substrate for the synthesis of other neurosteroids. The avian brain expresses the necessary enzymes for the conversion of progesterone to androgens and other metabolites (Tsutsui, 2011). For example, the progesterone metabolite allopregnanolone reduces aggressiveness in mammals (Pinna et al., 2003).

An exposure to a 10-min STI during the non-breeding season increased corticosterone in both blood and brain. In contrast, a 30-min STI did not affect corticosterone levels in either brachial vein or jugular vein plasma (Newman and Soma, 2011). The duration of the social stimulation might be important, as there are energetic consequences of prolonged aggression (Haller, 1995). Elevated glucocorticoids can be beneficial for short periods but potentially deleterious for prolonged periods (Grissom and Bhatnagar, 2009). This is especially relevant during the non-breeding season, which has several ecological challenges (e.g. low food supply, decreased daylength for foraging, low temperatures) and the costs of prolonged elevated corticosterone would be more pronounced. The role of glucocorticoids in non-breeding aggression has been studied in other species. Non-breeding Siberian hamsters are highly aggressive. In hamsters, adrenalectomy decreases aggression, whereas adrenal demedullation has no effect on aggression, suggesting that adrenocortical steroids promote non-breeding aggression (Demas et al., 2004). Although chronic cortisol administration does not affect aggression in Siberian hamsters (Scotti et al., 2015), cortisol levels in several brain regions are negatively correlated with aggression in non-breeding males (Munley et al., 2021).

The present data show that the non-breeding brain synthesizes T, which could be either from circulating or neural progesterone or de novo from cholesterol. This local synthesis is
rapidly modulated in response to a social challenge. Despite the lack of T in the circulation, both CON and STI had subjects had T in the brain which could be due to increased 3β-HSD activity during the non-breeding season (Pradhan et al., 2010). Furthermore, in contrast to blood T levels, neural T levels were rapidly modulated by the social interaction. Neural 3β-HSD activity in non-breeding song sparrows rapidly increases after an STI (Pradhan et al., 2010) which could explain the increase in brain T observed here. These rapid changes in brain 3β-HSD activity can be accompanied by rapid changes in brain aromatase activity that also occur in aggressive contexts (Black et al., 2005) to quickly modulate local estradiol in the brain.

The up regulation of T we observed in particular brain regions could play an important role in the modulation of non-breeding aggression, at least indirectly. Although castration does not decrease non-breeding aggression in male song sparrows, T administration increases particular aspects of aggression in non-breeding males (Wingfield, 1994). Moreover, in the non-breeding season, androgen receptors are expressed throughout the SDMN (Wacker et al., 2010). However, androgen receptor antagonism has no effect on aggression in short day housed (non-breeding like) song sparrows (Sperry, Wacker and Wingfield, 2010), suggesting that the role of T in non-breeding aggression might not be mediated through its action on androgen receptors.

An alternative is that in the non-breeding season, T can also serve as a substrate for estradiol synthesis which can in turn promote aggression. Aromatase, the enzyme that converts androgens to estrogens, is highly expressed throughout the SDMN in non-breeding song sparrows (Soma et al., 2003; Wacker et al., 2010). Interestingly, aromatase inhibition decreases aggression in non-breeding free living song sparrows (Soma et al., 2000; Soma, Tramontin and Wingfield, 2000), effects that are rescued by estradiol replacement (Soma, Tramontin and Wingfield, 2000). Moreover, estradiol administration increases aggression within 20 min only in
non-breeding season (Heimovics, Ferris and Soma, 2015). In mice, estradiol administration also rapidly promotes aggression in short day (non-breeding like) but not long day (breeding like) housed males (Trainor et al., 2007; Trainor, Sima Finy and Nelson, 2008). Taken together, these studies indicate that sex steroids synthesized in the brain are crucial for the regulation of non-breeding aggression. Although estradiol was not detected in non-breeding song sparrows in basal conditions (Jalabert, Ma and Soma, 2021), future studies should assess the effects of an STI in estradiol brain levels.

Non-breeding subjects do not have detectable 5α-DHT in the blood nor the brain. In a previous study, we also did not detect 5α-DHT in unchallenged song sparrows during the non-breeding season (Jalabert, Ma and Soma, 2021). Further, brain 5α-reductase activity is lowest during the non-breeding season (Soma et al., 2003). Lastly, given that AR antagonism does not affect non-breeding aggression in song sparrows (Sperry, Wacker and Wingfield, 2010), 5α-DHT synthesis is not expected to increase in response to STI in non-breeding subjects.

4.4.4 Seasonality of neuroendocrine mechanisms

Wild male song sparrows during the breeding and non-breeding seasons were robustly aggressive in response to the 10-min STI. Although aggression displayed in both seasons is very similar, the context is not the same. During each season, animals compete for access to different types of resources. Moreover, different environmental conditions cause distinct physiological states, and therefore, different neuroendocrine mechanisms regulate behavior depending on the context. The neuroendocrine response to the STI in each season is very different. In the breeding season, only glucocorticoids are rapidly modulated in circulation and brain. In contrast, in the non-breeding season, besides the increase on corticosterone, there is a rapid modulation of sex
steroids, principally in the brain. Steroid signaling can be regulated by several mechanisms including peripheral and local synthesis, inactivation and metabolism, blood-brain barrier passage, and sensitivity of neural circuits by modifying receptor abundance. Although it is probable that more than one of these mechanisms act simultaneously, the present data suggests that rapid neurosteroid synthesis plays a key role in the regulation of aggression especially in the non-breeding season.

Sex steroids, such as progestogens, androgens, and estrogens, act on the brain principally through two mechanisms. Long-lasting effects of steroids are linked to their actions by binding to their intracellular receptors to act as transcription factors and regulate gene expression in brain cells. These genomic effects of steroids on the central nervous system require several hours or days to develop and produce persistent changes in physiology and behavior. In addition, sex steroids can exert short-term, non-genomic effects, which are often mediated by plasma membrane receptors or by the allosteric modulation of neurotransmitter receptors. These rapid effects are typically seen within 30 min. Both mechanisms regulate aggression. In mice (*Peromyscus polionotus*) housed under “breeding-like” days, estradiol reduces aggression via genomic mechanisms; whereas under “non-breeding-like” days, estradiol rapidly (within 15 min) increases aggression via non-genomic mechanisms (Trainor *et al.*, 2007). Thus, sex steroids act via genomic or non-genomic mechanisms to regulate aggression throughout the year and the mechanism of action depends on the season.

The switch of mechanisms is crucial in changing environments. Sustained high levels of circulating steroid hormones, such as androgens, carry significant disadvantages for individuals. In birds, T implantation decreases body mass and fat reserves (Ketterson *et al.*, 1991; Wikelski *et al.*, 1999) and suppresses the immune system (Casto, Nolan V. and Ketterson, 2001). Therefore,
although aggression plays a crucial role to secure resources, such as food and shelter, it is especially important to maintain low levels of circulating steroids in the non-breeding season, at a time when ambient temperatures are low, photoperiods are short, and food is scarce.

4.5 Conclusions

In the present study, we show that steroid levels are differentially regulated within multiple brain regions in breeding and non-breeding adult male song sparrows. A social challenge rapidly modulated sex steroids in the non-breeding brain season only. These data, together with previous results, suggest that neurosteroids are rapidly synthesized in the brain in response to social cues when circulating sex steroid levels are low. This seasonal switch between neuroendocrine mechanisms would avoid the costs of chronically elevated systemic levels of steroids in wild animals.
4.6 Tables and figures

Figure 4.1: Effect of a 10 min simulated territorial intrusion (STI) on aggressive responses of wild adult male song sparrows during the breeding and non-breeding seasons

Bar graphs represent (A) response, (B) song, and (C) flight latencies, for control and STI subjects. Values are expressed as the mean ± SEM. n=10 per breeding group, n=9 per non-breeding group. **** p ≤ 0.0001.
Figure 4.2: Effect of a 10 min simulated territorial intrusion (STI) on aggressive responses of wild adult male song sparrows during the breeding and non-breeding seasons

Bar graphs represent (A) time in 5 meters, (B) number of songs, and (C) number of flights, for control and STI subjects. Values are expressed as the mean ± SEM. n=10 per breeding group, n=9 per non-breeding group. * p ≤ 0.05, ** p ≤ 0.01, **** p ≤ 0.0001.
Figure 4.3: Effect of a 10 min simulated territorial intrusion (STI) on circulation and brain steroid levels of wild adult male song sparrows during the breeding season

Bar graphs represent concentrations of (A) progesterone, (B) corticosterone, (C) testosterone, and (D) 5α-dihydrotestosterone, for control and STI subjects. Values are expressed as the mean ± SEM. n=10 per breeding group, n=9 per non-breeding group. # 0.1 < p < 0.05, * p ≤ 0.05, ** p ≤ 0.01, *** p≤0.001. nd, non-detectable
Figure 4.4: Effect of a 10 min simulated territorial intrusion (STI) on circulation and brain steroid levels of wild adult male song sparrows during the non-breeding season

Bar graphs represent concentrations of (A) progesterone, (B) corticosterone, and (C) testosterone, for control and STI subjects. Values are expressed as the mean ± SEM. n=10 per breeding group, n=9 per non-breeding group. # 0.1 < p < 0.05, * p ≤ 0.05, ** p ≤ 0.01, **** p ≤ 0.0001. nd, non-detectable.
Chapter 5: Conclusion

Aggression is an important social behavior that plays key roles in obtaining and maintaining access to limited resources. Traditionally, gonadal steroids have been the primary focus of neuroendocrine studies on aggression. While gonadal steroid secretion is important in modulating aggression, recent work indicates that the brain itself can produce steroids and metabolize circulating steroids to act locally and modulate behavior.

In this dissertation, I have presented three studies in adult male song sparrows that examined the hypotheses that the brain regulates local steroid levels throughout the year and that aggressive interactions rapidly stimulate neurosteroid synthesis in the brain only during the non-breeding season. I found that 1) brain steroid levels greatly differed from circulating steroid levels, 2) steroids showed very different seasonal patterns in the blood and brain and the brain:blood ratio can be reversed between seasons, and 3) adult male song sparrows are highly aggressive in both seasons but the neuroendocrine response to social challenges was very different between breeding and non-breeding animals. Taken together, these data suggest that in the adult male song sparrow neurosteroids are locally regulated within the brain and neurosteroid levels are rapidly modulated by social interactions in a season- and region-specific manner.

5.1 Major findings

5.1.1 Local regulation

There is a growing body of evidence that the brain can actively regulate local steroid levels, in contrast to the traditional view of the brain as a passive recipient of steroids produced in the periphery (e.g., by the gonads). In birds, several mechanisms regulate local steroid levels.
in the brain, including blood-brain barrier passage (Diotel et al., 2018), steroid binding proteins (Lin et al., 2021), local synthesis (Tsutsui, 2011), and local metabolism (Hutchison, 1991).

I provide further evidence for local steroid regulation in the song sparrow brain. In Chapters 2, 3, and 4, all steroids measured showed different concentrations across the brain areas studied. In chapters 2 and 4, both progesterone and corticosterone levels were higher in blood than in brain. However, in Chapter 4, increases in progesterone or corticosterone in blood did not directly correspond with increases of those steroids in brain, indicating the precise regulation of steroids in brain could be either by passage to the brain or by local metabolism. Further, in Chapters 2 and 4, breeding animals showed higher 5α-DHT levels in brain than in blood. Similarly, in Chapters 2 and 3, estrogens were high in some brain regions, despite levels in circulation being non-detectable or very low. Interestingly, T levels were higher in blood than in brain of breeding animals. T is a substrate of 5α-reductase and aromatase to synthesize 5α-DHT and 17β-E2 respectively. Therefore, high levels of circulating T might provide substrate for neural synthesis of potent androgens or estrogens. The studies presented here further support that blood steroid measurements often do not reflect the steroid levels within tissues and that steroid measurement in discrete samples provide a better understanding of local steroidal environments. Future studies should seek to understand the mechanisms by which the brain modulate local steroid levels in these contexts.

### 5.1.2 Rapid local steroid production

While gonadal steroid secretion is important in modulating aggressive behaviors, recent work indicates that these behaviors can be regulated by steroids produced in the brain. A growing body of evidence supports local steroid production in the songbird brain including: the
presence of all necessary steroidogenic enzymes within discrete brain regions as shown by gene expression and \textit{in situ} hybridization assays (London, Boulter and Schlinger, 2003; Soma \textit{et al.}, 2003; London \textit{et al.}, 2006; Wacker \textit{et al.}, 2010), steroid production in brain tissue \textit{in vitro} (Soma \textit{et al.}, 2003, 2004; London \textit{et al.}, 2006; Pradhan, Yu and Soma, 2008; Pradhan \textit{et al.}, 2010), increase of steroid levels within the brain during social interactions \textit{in vivo} (Remage-Healey, Maidment and Schlinger, 2008; Remage-Healey \textit{et al.}, 2012), and detectable levels of steroids in the brain of gonadectomized animals (Matsunaga, Ukena and Tsutsui, 2002; Liere \textit{et al.}, 2019). In song sparrows, rapid (< 10 mins) steroid production in the brain during an aggressive challenge seems to be particularly important during the non-breeding season (Soma, 2006; Heimovics, Fokidis and Soma, 2013).

Here, we found that an aggressive interaction induces region-specific changes in concentrations of progesterone, glucocorticoids, and androgens in the male song sparrow brain particularly in the non-breeding season. Interestingly, although progesterone increased in the blood of STI animals, its local regulation in the brain is more precise as we saw an increase, decrease, or no change in local progesterone levels depending on the brain region. Importantly, T was not detectable in blood of either CON or STI animals, while an aggressive interaction induced rapid changes in specific brain regions. In contrast, the neuroendocrine response to the social challenge in the breeding season was very different as only glucocorticoids were rapidly modulated in blood and brain. These data, when considered together with previous work, suggest that neurosteroids are rapidly synthesized in the brain and play a key role in the modulation of social behavior, particularly when circulating steroid levels are low. These results support that the brain can fine tune neural steroids, and that neurosteroids have a role in sustaining aggressive behavior in the non-breeding season when environmental conditions are especially challenging.
and the costs of high levels of circulating steroid hormones could be crucial. The seasonal switch between mechanisms would avoid the costs of chronically elevated systemic levels of steroids in wild animals.

5.2 Additional contributions

5.2.1 Liquid chromatography tandem mass spectrometry for steroid profiling

Immunoassays have been widely used to measure steroids due to their technical simplicity, high sample throughput, and easy sample preparation. However, compared to LC-MS/MS, immunoassays have potential limitations, including lack of sensitivity for measuring steroids in small sample amounts, measurement of single analytes, and most importantly, antibody cross-reactivity with similar analytes resulting in an overestimation of steroid concentrations. In chapter 2, we developed and validated a LC-MS/MS assay for the measurement of 10 steroids in a single small sample. We used our assay to measure corticosterone, progesterone, androgens, and estrogens in wild animals to better understand the balance between peripheral and brain steroid levels across seasons. Thus, with our approach, a much broader understanding is gained of the systemic and local steroidal environments, relative to a narrow focus on a few steroids just in the circulation. Finally, our LC-MS/MS protocol for steroid profiling is applicable to other sample types and species and will be broadly useful for neuroendocrinology studies.
5.2.2 Ultra-sensitive liquid chromatography tandem mass spectrometry assay for estrogen measurement

Estrogens are a large class of steroids that can be locally synthesized within the brain and play key roles in multiple neural functions such as regulation of social behavior, learning and memory, and cognition. However, measurement of estrogens in specific brain regions is extremely challenging given that they are present at very low endogenous levels, and therefore, assay sensitivity is a major factor to measure them in biological samples. Further, the brain has a large amount of lipids that can interfere with steroid measurement, and therefore, assays can suffer from matrix effects when measuring estrogens in the brain. In chapter 3, we developed a method using liquid chromatography tandem mass spectrometry combined with derivatization to increase sensitivity for the measurement of E1, 17β-E2, 17α-E2, and E3 in microdissected brain, plasma, and blood in a songbird model. This technique will be useful for measurement of estrogens in songbirds and other animal models that will facilitate studies of neuroestrogens and their functions.

5.3 Limitations and future directions

As shown by the data presented here, local steroid levels can greatly differ among brain regions. Further, discrete brain regions within the SDMN play different roles in different social contexts. Therefore, steroid measurement in microdissected brain tissue provides a better understanding of local steroid signaling. However, due to the limited amount of tissue obtained by microdissection (1-2mg), the low endogenous levels at which some sex steroids exert their actions, and the non-breeding samples in which steroid levels are even lower, we had several...
samples that were below the detection limit of the assay. Therefore, there is a trade-off between spatial resolution and obtaining detectable quantities of steroids.

The analysis of non-detectable samples constitutes a challenge. The actual steroid level for a non-detectable sample is a value between zero and the detection limit of the assay. In endocrinology studies, there have been different substitution methods for those non-detectable values (missing not at random), including setting them at the detection limit, the detection limit/2, the detection limit/√2, zero, or just deleting the sample. These different methods add a different degree of bias, from lowering the group mean and standard deviation (when substituting by zero) to increasing them (when deleting the sample), being detection limit/√2 the best method for reducing bias. However, all of these methods increase bias as more samples are substituted and with smaller sample size, especially for correlational analysis (Handelsman and Ly, 2019). Further, all aforementioned methods substitute all the non-detectable (missing not at random) values with the same number and therefore there is an increased chance to violate the principle of homogeneity of variances for parametric statistical analyses. When a proportion (e.g. 20%) of the samples within a group are detectable, then the missing value imputation approach can be used to obtain normal distribution for missing elements below the detection limit (Wei, Wang, Jia, et al., 2018; Wei, Wang, Su, et al., 2018). This method uses the lowest observed value as input to establish the upper truncation point (i.e., the detection limit of the assay) and then uses predictive information from detectable samples in the group as well as other variables to impute missing values. The result is a normal distribution for the imputed values based on the existing data within a reasonable limit. This method has shown to be the most effective method for a larger number of missing samples introducing minimum bias for statistical analysis.
The derivatization method developed in chapter 3 is a step forward to increase the sensitivity in estrogen analysis. However, the current method has limitations. We were not able to distinguish between 2OH-E2 and 4OH-E2 analytes, as they share transitions and retention times. Future studies could modify the LC to overcome co-elution of these analytes. Also, 4OH-E2, 2Me-E2, and 4Me-E2 analytes suffered from matrix effects. Future studies can include additional extraction steps or additional IS for catechol and methoxy estrogens to correct for matrix effects.

In chapter 4, I found that in the non-breeding season, aggressive interactions induced rapid changes of T concentrations in the brain while levels were not detectable in the general circulation. These data suggest that T is being rapidly synthesized in the brain of STI animals. However, estrogens, rather than androgens per se, seem to be critical modulators of non-breeding aggression (Soma et al., 2000; Soma, Tramontin and Wingfield, 2000; Heimovics, Ferris and Soma, 2015). In chapters 2 and 3, in the non-breeding season, neuroestrogen levels are very low at baseline. Future studies should assess the effects of an STI on estrogen levels in blood and brain with the sensitive method developed in chapter 3.

When comparing steroid levels in the brain and the circulation, there are at least two key aspects to consider. First, most studies measure steroid concentrations in serum or plasma to evaluate the levels of steroids to which the brain is exposed to. However, since plasma steroid concentrations are about twice as high as blood steroid concentrations (Taves et al., 2010, 2011; Tobiansky et al., 2018; Jalabert, Ma and Soma, 2021), plasma measurements can overestimate the steroid levels that embedded tissues. Therefore, measuring levels in the blood provides a better understanding of the steroidal environment to which tissues are exposed. Second, steroids in the circulation are bound to carrier proteins (e.g. in blood about 90% of corticosterone is
bound to CBG with high affinity), and thus the passage of circulating steroids into the tissues can also be limited to the free fraction. Further, CBG levels can fluctuate along the seasons as well as after social interactions (Romero 2002, Charlier 2009) which will alter steroids that would reach the brain at different times. Therefore, measuring free (unbound) steroid levels would be a better representation of the steroids that could reach the brain. In the studies presented here, we measured total steroid levels (free + bound). Future studies should measure both free and bound fractions to better understand steroid levels in the blood.

Local levels of steroids are regulated by several mechanisms, including production and metabolism/inactivation. Metabolizing enzymes that catalyze steroid inactivation are key to terminate steroid action. In the quail, levels of 5β-DHT (the inactive T metabolite) are much higher than those in the circulation and about 10 to 20 times higher than brain T, which suggests that T is being highly locally inactivated (Liere et al., 2019). In song sparrows, 5β-reductase (the enzyme that converts T to 5β-DHT) activity is elevated in the ventromedial telencephalon and NCM in breeding males (Soma et al., 2003) which probably reflects elevated inactivation when T levels are high. Reduced 5β-reductase activity in non-breeding males could be a mechanism to maintain androgen levels in the brain and increase sensitivity to androgens. Future studies should look at the enzymes along the steroidogenesis and metabolism pathway in response to an aggressive interaction in order to understand the production inactivation balance by which the brain modulate local steroid levels.

The lack of STI effect on sex steroids during the breeding season might be related to the duration of the aggressive challenge we employed. Circulating T levels increase following 10-30 min STI in wild male song sparrows (Wingfield, 1985; Wingfield and Wada, 1989), brain 17β-E₂ levels in microdissected punches are modulated after a 30-min STI in wild male white-crowned
sparrows (Charlier et al., 2011), and in vivo microdialysis studies show that when males are exposed to other males’ song local 17β-E2 levels increase while T levels decrease in the NCM of zebra finches (Remage-Healey, Maidment and Schlinger, 2008). Future studies should examine the effects of STI on longer time points (e.g. 30 min) employing in brain microdissection or in vivo microdialysis followed by steroid profiling via LC-MS/MS to evaluate the effect of an aggressive social challenge on neurosteroidogenesis in breeding contexts.

In chapter 4 we saw interesting data regarding progesterone changes following an STI. In non-breeding animals, progesterone increased in the circulation while in the brain there was a region-specific response. In contrast, during the breeding season, progesterone levels were not modulated by STI which might be related to the timing of the aggressive challenge. In female song sparrows, progesterone levels in the circulation do not change after an STI in breeding or non-breeding seasons (Elekonich and Wingfield, 2000). However, little attention has given to the role of progesterone on aggression in male song sparrows. In other species, the relationship between progesterone and aggression has mostly studied in breeding contexts where it seems to play important roles. Progesterone could regulate non-breeding aggression by either modulating progesterone receptor activation or by local conversion to androgens and estrogens that would in turn modulate non-breeding aggression. Future studies should examine the potential mechanisms by which progesterone might modulate aggression in breeding and non-breeding song sparrows.

These studies focus only on males, but studies of females are needed to make generalizations. Although many aspects of song sparrow female aggression appear to be similar to male aggression, the intensity and persistence of aggression in females seems to be lower than in males (Elekonich and Wingfield, 2000) and also females rarely sing (Arcese, Stoddard and Hiebert, 1988). Further, wild female song sparrows are aggressive mainly during pre-breeding
and breeding seasons whereas non-breeding aggression is low (Elekonich and Wingfield, 2000).
Therefore, the seasonal variation and endocrine mechanisms that regulate aggression would be
different between the sexes and female studies should be conducted to have a better
understanding of the regulation of neurosteroids in this species.

5.4 Conclusions

In this dissertation, I present evidence for local regulation of steroid levels within the
nervous system. To my knowledge, these are the first data to describe natural seasonal changes
in brain steroid levels with great spatial resolution in any species. Further, this is the first study to
measure a large panel of sex steroids such as T, 5α-DHT and 17β-E₂ as well as their precursors
in songbirds providing a comprehensive picture of the steroidal environments in the circulation
and specific brain regions. Finally, this is the first report of rapid local modulation of steroids
within discrete brain regions that regulate social behavior in response to a social challenge in
wild songbirds. Together, these studies demonstrate local steroid regulation in the brain and
regulation by season and social context. When considered together with previous work, these
data suggest that neurosteroids are rapidly synthesized to regulate aggression when gonadal
steroid synthesis is low. Thus, neuroendocrine mechanisms switch between seasons from
peripheral to neural to support year-round aggression avoiding the costs of maintaining elevated
systemic steroid levels. Finally, as steroid levels are locally regulated within different brain
regions and can greatly differ from steroid levels in the circulation, this work highlights the
importance of measuring levels in tissues to have a precise understanding of local steroid action.
Bibliography


Camacho-Arroyo, I. et al. (2007) ‘Changes in progesterone receptor isoforms content in the brain


Goodson, J.L. (2005) ‘The vertebrate social behavior network: Evolutionary themes and


Hamden, J.E. *et al.* (2019) ‘Measurement of 11-dehydrocorticosterone in mice, rats and


Remage-Healey, L. et al. (2017) ‘Rapid Effects of Estrogens on Avian Brain and Social


Soma, K.K. et al. (2003) ‘Brain aromatase, 5α-reductase, and 5β-reductase change seasonally in


Trainor, B.C. *et al.* (2007) ‘Photoperiod reverses the effects of estrogens on male aggression via


doi:10.1016/j.neuron.2017.06.046.