DEHYDROEPIANDROSTERONE AND 17BETA-ESTRADIOL IN PLASMA AND BRAIN OF DEVELOPING AND ADULT ZEBRA FINCHES

by

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Abstract

The classical model of sexual differentiation states that genes influence gonadal differentiation, and gonadal hormones then drive sexual differentiation throughout development. This model has been called into question by research, especially in songbirds, providing evidence for alternative mechanisms like direct effect of genes and local production of steroids via *de novo* synthesis or local metabolism of steroid precursors like DHEA, which can be metabolized to testosterone and E2. In order to assess the role of local steroid production on sexual differentiation in songbirds, levels of DHEA and E2 were measured in brachial and jugular plasma, as well as brain and peripheral tissues in zebra finches at critical ages during development and in adulthood. DHEA levels in brachial and jugular plasma peaked at P30 and higher DHEA levels in jugular plasma were found in males relative to females at P30. Also, at P30, higher DHEA levels were found in rostral telencephalon in females relative to males. The findings of this study indicate that DHEA may play a role in sexual differentiation of songbirds. Surprisingly, E2 was non-detectable in many plasma and tissue samples. Higher E2 was found in the diencephalon in females relative to males at P3/P4 and higher E2 was found in gonads in adult females relative to males. There was little evidence to suggest that E2 is synthesized *de novo* in the brain, although perhaps E2 is being rapidly metabolized into another estrogen or E2 synthesis is more localized in the synapase. The findings of this study support the role of alternative mechanisms like *de novo* steroid synthesis and local metabolism of steroid precursors and challenge the role of classical mechanisms of sexual differentiation in songbirds. Also, these findings may have important implications for sex differences, which develop independently of gonadal hormones, in other animal species.
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### Abbreviations

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<tr>
<td>E₂: 17β-estradiol</td>
<td>Estradiol</td>
</tr>
<tr>
<td>DHEA:</td>
<td>Dehydroepiandrosterone</td>
</tr>
<tr>
<td>P:</td>
<td>Post-hatch day</td>
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<td>ED:</td>
<td>Embryonic day</td>
</tr>
<tr>
<td>SNB:</td>
<td>Spinal nucleus of bulbocavernosus</td>
</tr>
<tr>
<td>MePD:</td>
<td>Posterodorsal medial amygdala</td>
</tr>
<tr>
<td>T:</td>
<td>Testosterone</td>
</tr>
<tr>
<td>AE:</td>
<td>Androstenedione</td>
</tr>
<tr>
<td>SDN-POA:</td>
<td>Sexually dimorphic nucleus of the preoptic area</td>
</tr>
<tr>
<td>SRY:</td>
<td>Sex-determining region of the Y chromosome</td>
</tr>
<tr>
<td>ER:</td>
<td>Estrogen receptor</td>
</tr>
<tr>
<td>AR:</td>
<td>Androgen receptor</td>
</tr>
<tr>
<td>DHT:</td>
<td>Dihydrotestosterone</td>
</tr>
<tr>
<td>TEL:</td>
<td>Telencephalon</td>
</tr>
<tr>
<td>rTEL:</td>
<td>Rostral telencephalon</td>
</tr>
<tr>
<td>dTEL:</td>
<td>Dorsal telencephalon</td>
</tr>
<tr>
<td>cTEL:</td>
<td>Caudal telencephalon</td>
</tr>
<tr>
<td>OL:</td>
<td>Optic lobe</td>
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<tr>
<td>DIEN:</td>
<td>Diencephalon</td>
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Co-authorship Statement

I collected plasma and tissue, extracted steroids, measured steroids, analyzed data and wrote the manuscript. Eunice Chin and Kim Schmidt assisted with plasma and tissue collection. Mark Huang helped with sex genotyping and steroid extractions and lastly, Amy Newman helped with modifying the E2 assay.
1. Classical and Novel Mechanisms for Sexual Differentiation of Brain and Behavior

1.1 Introduction

Sex differences in behavior are prevalent in many animal species, including humans, and have been extensively studied by researchers from different perspectives. At an evolutionary level, these differences may be explained by sexual selection, which refers to the competition for mates that occurs within sexes (Darwin, 1871). At a proximate level, a common strategy to explain these sex differences is to map behavioral differences onto sex differences in brain structure or function. This has revealed sex differences in the size of every brain lobe relative to cerebrum volume and very large sex differences in the structure and function of the hippocampus and amygdala, in humans (reviewed in Cahill, 2006). However, much of our current knowledge about sexual differentiation, the process by which males and females develop sex differences from an undifferentiated zygote, was obtained from studying animal models.

Our current understanding of sexual differentiation comes largely from the finding by Alfred Jost (1970) that the absence of gonads in rabbits promotes female-typical development, regardless of the genetic sex. This led to the formulation of the now classic model of sexual differentiation stating that genetic sex, which is determined at fertilization, determines gonadal fate, and gonadal hormones then drive sex differences throughout development. Later research by Phoenix and colleagues (1959) on guinea pigs showed that hormones can have effects during critical periods in early development (organizational effects) or during adulthood (activational effects). The subsequent study of several sexually dimorphic model systems in rodents such as the sexually dimorphic nucleus of the preoptic area (SDN-POA), spinal nucleus of the bulbocavernosus (SNB), and posterodorsal medial amygdala (MePD) area indicates that gonadal hormones are responsible for driving sex differences in these systems (reviewed in Morris et al.,
Testosterone (T) is aromatized to 17β-estradiol (E2) in the brain to induce a masculine SDN-POA, while T acts directly on the SNB and MePD by binding to androgen receptors (ARs). While these sex differences fit the classical model of sexual differentiation, this model fails to account for sex differences observed before gonadal development in mammals and sex differences in songbirds.

1.2 Exceptions to the Classical Model of Sexual Differentiation

1.2.1 Mammals

Recent research has shown that sex differences exist in mammals before gonadal development, which occurs at approximately embryonic day 12 (ED12) in rodents and six weeks in utero in humans. For instance, higher embryonic growth rates in genetic males have been found as early as ED3.5 in rats (Burgoyne, 1993). This finding was replicated in pre-implanted human embryos that were fertilized in vitro (Pergament et al., 1994). Additionally, sex differences have been found after gonadal differentiation but before gonadal hormone synthesis, which occurs at approximately ED15 in rodents and 12-17 weeks in utero in humans. For instance, in rat embryonic mesencephalic or diencephalic neurons cultured at ED14, females express more tyrosine hydroxylase, an enzyme involved in catecholamine synthesis, than males (Reisert and Pilgrim, 1991). However, this sex difference is not regulated by the sex-determining region of the Y chromosome (SRY) gene (Carruth et al., 2002) and may instead be regulated by other genes on the Y chromosome or genes on the X chromosome that escape inactivation (see section 1.3.1). Taken together, these studies suggest that sex chromosomes influence embryonic growth rates and enzyme expression immediately after fertilization and prior to gonadal hormone production.
Another well-studied exception to the classic model of sexual differentiation is found in songbirds. In many songbird species, males sing to court females and defend territories, whereas females sing less. The sexual dimorphism of song behavior is most extreme in songbird species like zebra finches (Taeniopygia guttata), where males sing and females do not. Correspondingly, pronounced sex differences were found by Arnold and Nottebohm (1976) in the song nuclei controlling song behavior in zebra finches. These sex differences are still one of the largest ones observed in the brain of any species (reviewed in Ball and MacDougall-Shackleton, 2001).

Song learning in zebra finches occurs in two overlapping phases. In the sensory learning phase, which extends from P15 to P60, zebra finches listen and store into memory the songs they hear. In the sensorimotor learning phase, which extends from P25 to P90, zebra finches actively practice singing. Song crystallization occurs at P90, which corresponds with the age when zebra finches typically have a complete song that remains relatively stable. Developing zebra finches are particularly sensitive to hormones between P0 and P40 (Konishi and Gurney, 1982).

Administering E2 to neonatal female zebra finches and T when they reached adulthood induced the development of male-typical song behavior and nuclei (Gurney, 1981; Gurney and Konishi, 1980; Simpson and Vicario, 1991a; Simpson and Vicario, 1991b). In contrast, treating young males with estrogen receptor (ER) antagonists (Mathews and Arnold, 1990; Mathews and Arnold, 1991; Mathews et al., 1988) or aromatase inhibitors (Wade and Arnold, 1996; Wade et al., 1996) did not affect song system development, providing conflicting evidence for the role of E2 in masculinizing the song circuit.
Experiments attempting to determine the source of sex steroids during development indicate that it is not the gonads. For instance, gonadectomized young male zebra finches at post-hatch day 7-9 (P7-9; P0 is defined as day of hatch) and found that this did not affect the development of the song system (Adkins-Regan and Ascenzi, 1990; Arnold, 1975), but song developed more slowly in castrated males than control males (Arnold, 1975). Wade and colleagues (1996) induced functional testes in neonatal genetic females by administering an aromatase inhibitor and found that song system development was not affected. Additionally, several different approaches to establishing sex differences in developmental exposure to hormones have not provided consistent results (see below), further supporting the argument that classical mechanisms of sexual differentiation do not apply to songbirds. Clearly, a different model is needed to explain sexual differentiation in songbirds and alternative sources of sex steroids need to be examined.

### 1.2.2.1 Circulating Steroids

Several studies have measured sex steroid levels in the plasma of young male and female songbirds. The earliest attempt by Hutchison et al. (1984) found that males had much higher (~9X) E$_2$ levels than females on P3 and that females had higher levels of androstenedione (AE), a precursor of T, than males on P1 and P9. A subsequent study by Adkins-Regan and colleagues (1990) found an overall effect of sex (F>M) on plasma T concentrations between P1 and P54 (no sex differences on individual days) but no effect of sex on plasma E$_2$ or dihydrotestosterone (DHT), a non-aromatizable androgen, concentrations. However, they found that males had higher plasma E$_2$ levels on P12. Lastly, Schlinger et al. (1992) found non-detectable plasma E$_2$ levels between P1 and P13, and no sex difference in plasma levels of estrone, DHT, or AE. The inconsistencies between the studies may be partially explained by differences in plasma source.
Hutchison et al. (1984) sampled jugular plasma, which is enriched with steroids from the brain (Schlinger and Arnold, 1993); Adkins-Regan et al. (1990) sampled jugular plasma in birds younger than P4 and brachial plasma in P4 and older birds; Schlinger et al. (1992) sampled plasma via cardiac puncture. Inconsistencies may also be explained by differing cross-reactivities and sensitivities of the different antibodies used in each of the three studies.

Steroid levels have also been measured in adult zebra finches. No sex differences have been found in adult levels of E2 or DHT (Adkins-Regan et al., 1990; Hutchison et al., 1984). However, higher levels of T were found in adult females relative to males (Hutchison et al., 1984).

1.2.2.2 Steroid Receptors

Other studies have examined steroid receptors in song nuclei regions of the brain to determine sex differences during development. Few neurons with ER have been found in song nuclei of developing males and females, with the exception of HVC (Gahr and Konishi, 1988; Nordeen et al., 1987). However, no obvious sex difference in ER mRNA was detected in the brain during embryonic and post-hatch development (Jacobs et al., 1999; Perlman and Arnold, 2003). Interestingly, higher androgen receptor (AR) mRNA expression was found in male zebra finches in important song nuclei during early development at P7-11 (Perlman et al., 2003). Furthermore, AR expression was upregulated in hatchling females treated with E2 and downregulated in hatchling males treated with aromatase inhibitor, suggesting that E2 masculinizes AR expression during development (Kim et al., 2004). Blocking ARs with flutamide prevents masculinization of the song circuit in young females treated with E2 (Bottjer and Hewer, 1992), indicating that androgens play a role alongside E2 in masculinizing the song nuclei. However, there is also conflicting evidence for the role of androgens in sexual
differentiation of songbirds. For instance, DHT only has minor masculinization effects on song nuclei that are much less than those of E$_2$ (Gurney, 1981; Schlinger and Arnold, 1991) and aromatizable androgens are not more effective than E$_2$ alone in masculinizing females (Grisham and Arnold, 1995). Also, long-term treatment with flutamide, from P7 to P61-63, only led to minor demasculinization of song nuclei in males (Grisham et al., 2007).

In addition to finding sex differences in receptor expression, recent studies have also examined expression of steroid receptor coactivators, which increase transcriptional activity of intracellular steroid receptors. For instance, Duncan and Carruth (2007) found higher mRNA expression of L7/SPA, an estrogen coactivator, in the brain of P1 and adult birds and higher protein expression of this coactivator in the male brain at P1, P10, P30, and adulthood.

1.2.2.3 Steroidogenic Enzyme Expression and Activity

Another approach for determining whether sex differences in hormonal exposure exist during songbird development is to measure expression and activity levels of enzymes that synthesize sex steroids. Aromatase mRNA expression is sexually monomorphic in song nuclei regions at P5, P10, P18, and P25 (Jacobs et al., 1999). Moreover, aromatase activity was not found to differ between males and females in whole brain homogenates of P4-6 or P10-12 birds (Schlinger and Arnold, 1992), micropunches of song nuclei regions in P20 birds (Vockel et al., 1990), and dissociated cell cultures from telencephalon of P0-1 birds (Wade et al., 1995). Other steroidogenic enzymes, including 3β-HSD, CYP11A1, and CYP17, are expressed in zebra finch brain during the first week of life but no sex differences in gene expression were found (London et al., 2003; London et al., 2006). Furthermore, the activity of 3β-HSD, which converts pregnenolone into progesterone and dehydroepiandrosterone (DHEA) into AE, was found to be
sexually monomorphic in homogenized brain regions from P5 birds (London et al., 2006), and in brain slices of P20 birds (Tam and Schlinger, 2007).

1.3 Alternative Mechanisms of Sexual Differentiation

1.3.1 Direct Genetic Influence

The “direct” role of genes on brain sexual differentiation is implicated in cases where sex differences are observed prior to gonadal development (see section 1.2.1). Microarray analysis in adult mice indicates that 650 genes (~14% of the genes expressed in the brain) are differentially expressed in the brains of males and females (reviewed in Davies and Wilkinson, 2006). More relevantly, recent studies have carried out similar analyses in animals prior to gonadal development. In mice at ED10.5 (gonadal differentiation begins at ~ED12), 36 genes were found to be expressed more in female brains while 18 genes were found to be expressed higher in male brains (Dewing et al., 2003). The majority of these genes play a role in cellular differentiation and proliferation, transcriptional regulation, and signaling processes.

In birds, females are the heterogametic sex (ZW) and males are the homogametic sex (ZZ). In chickens at ED4-10 (gonadal differentiation begins at ~ED6.5), 133 genes were differentially expressed in the brain and a majority of these genes were found to be differentially expressed at ED4 (Scholz et al., 2006). Interestingly, only 19 of those were expressed higher in females while the remaining 114 were expressed higher in males and found mainly on the Z chromosome, suggesting that genes may play an important role in sexual differentiation in songbirds. Clearly, sex differences in gene expression are present before gonadal development in vertebrates, suggesting that genes may “directly” contribute to sexual differentiation. However,
the role of differentially expressed genes on gonadally-independent sex differences is largely unknown.

A startling example of genetic influence on sexual differentiation is seen in a gynandromorphic zebra finch (Agate et al., 2003). A rare adult zebra finch was discovered with male plumage on the right half of the body and female plumage on the left half of the body. The bird exhibited masculinized song, male-typical courtship and copulatory behavior, plasma E2 levels that were below detection, and plasma T levels similar to control females. Moreover, the phenotype of the bird was lateralized in several tissues including gonads and brain. Since both halves were presumably exposed to the same level of circulating steroids during development, this lateralized phenotype is likely due to lateralized gene expression.

1.3.1.1 Mechanisms of Direct Genetic Influence

Genes on the Y chromosome are likely to be exclusively expressed in males, unless they have homologs that are also found on the X chromosome, and may directly contribute to sex differences. The \textit{SRY} gene is the best studied gene present on the Y chromosome because of the important role it plays in triggering testicular development from the undifferentiated gonad in genetic males (reviewed in Wilson and Davies, 2007). Interestingly, a circular form of the \textit{SRY} transcript (immature, untranslatable form) was detected in male brains of mice as early as E11 and a linear form (mature, translatable form) was detected postnatally in the diencephalon, midbrain, and cortex (Dewing et al., 2006). Additionally, in rats, \textit{SRY} is expressed in the substantia nigra in tyrosine hydroxylase-expressing neurons, and blocking \textit{SRY} expression in this region via antisense oligonucleotides dramatically reduces the number of tyrosine hydroxylase expressing neurons, leading to motor deficits (reviewed in Bocklandt et al., 2007). It is hypothesized that females, without \textit{SRY} expression in the brain, are able to maintain number of
tyrosine hydroxylase expressing neurons and motor function due to a compensating factor, such as E2, which is shown to influence tyrosine hydroxylase activity. Thus, differential gene expression may contribute to sex differences but these differences in genetic expression may also be compensated for by other factors, including hormones.

In mammals, females have two X chromosomes and to compensate for this, they undergo random inactivation of one of the X chromosomes during early development (reviewed in Arnold, 2004; Heard, 2004). However, some genes on the pseudoautosomal region of the X chromosome, which is common to both the X and Y chromosomes, do not undergo inactivation (~20% of X chromosome genes in humans) and may contribute to sex differences due to expression of both chromosomes. Microarray analysis in chickens indicates higher gene expression level of most sex-linked genes in males relative to females suggesting that dosage compensation is weak or absent in birds (Ellegren et al., 2007). It is hypothesized that a double dose of Z-linked genes in males may contribute to sexual differentiation. Support for this comes from the finding of differential expression of the Z-linked gene trkB, which is a receptor for the neurotrophic factors like BDNF that influence neuronal survival, migration and differentiation (Chen et al., 2005). TrkB mRNA is expressed at higher levels in males in the telencephalon and whole brain at P6 and adulthood. Importantly, protein levels of trkB were also higher in males, suggesting that dosage compensation is not occurring at the post-transcriptional level. Moreover, BDNF is found at higher levels in males than females in the telencephalon at P30 and is known to stimulated by E2 (Dittrich et al., 1999), suggesting that this Z-linked gene may be involved in sexual differentiation.
1.3.2 Local Steroid Production

Another potential mechanism for sexual differentiation is the local production of steroids in the brain. The aromatization of T into E₂ in the mammalian brain is clearly shown to contribute to sexual differentiation in mammals (reviewed in Lephart, 1996). However, the role of neurosteroids, which are steroids that are synthesized de novo, and the metabolism of other steroid precursors have not been thoroughly examined in the context of sexual differentiation (reviewed in Schlinger et al., 2001). In rats, higher levels of E₂ were found in the male frontal cortex, hypothalamus, and preoptic area but not in the hippocampus, cerebellum, and brain stem within 2 hours of birth (Amateau et al., 2004). At 32 hours after birth, higher E₂ levels were still found in male hypothalamus and preoptic area and now interestingly, higher E₂ levels were found in the female hippocampus. Higher E₂ levels in males and females declined after treatment with an aromatase inhibitor, suggesting that E₂ may be synthesized de novo in the female hippocampus since circulating levels of T are very low in developing females. In songbirds, some evidence supporting the role of neurosteroids in vertebrate sexual differentiation comes from studying cultured brain slices of male and female zebra finches at P25 (Holloway and Clayton, 2001). Masculine development of fiber growth from HVC to RA was stimulated in female brain slices co-cultured with male slices or with E₂ and this outgrowth of fibers was inhibited by ER antagonists. Furthermore, by comparing E₂ levels in brachial plasma (index of systemic steroid levels) and jugular plasma (indirect index of neural steroid levels) of European Starlings (Sturnus vulgaris), Chin and colleagues (2008) found that neural E₂ metabolism may be greater in males than females at specific ages during development. These sex differences in neurosteroid levels found in rodents and songbirds during early development may be involved in sexual differentiation but clearly, more research needs to be done on this hypothesis.
The role of sex steroid precursors like DHEA, which can be metabolized to T and E2, in sexual differentiation is poorly understood. Interestingly, DHEA sulfate (DHEAS), which can be converted into DHEA, is found at very high levels in the human fetus and on the day of birth, but drops precipitously afterwards (reviewed in Rainey and Nakamura, 2008). The role of high levels of DHEAS during early development is largely unknown. Part of the difficulty in studying this question is due to low systemic DHEA levels in common animal models like rodents (Baulieu and Robel, 1998; Labrie et al., 2005). In contrast, songbirds are an excellent alternative since they have high systemic DHEA levels (Soma and Wingfield, 2001). Interestingly, Migues and colleagues (2002) found higher DHEA levels in the forebrain region of P1 chickens relative to plasma levels, and higher levels in the female forebrain relative to male forebrain. DHEA levels in developing zebra finches are not known, but they have been studied in jugular and brachial plasma of European starlings (Chin et al., 2008). DHEA levels are higher in males than females at specific ages during development in starlings. The hypothesis that steroid precursors like DHEA are involved in sexual differentiation also needs to be further researched.

In adult zebra finch brains, aromatase expression is especially abundant relative to other vertebrate species, and is found to be most abundant in the synaptosomal fraction (Rohmann et al., 2007). Using immunoelectron microscopy, it was shown that aromatase is present in presynaptic boutons in the HVC, which have few aromatase-positive somata and high ER expression (Peterson et al., 2005). Moreover, males had higher aromatase expression in presynaptic boutons and more synapses than females. This suggests that synaptic aromatization may be a mechanism of delivering high levels of E2 directly at the synapse and may function in a sex-specific manner in adult songbirds. In contrast, juvenile zebra finches at P4-6 had higher aromatase activity in the microsomal relative to synaptosomal fraction of telencephalon
subcellular fractions, and no sex difference was found in either of the two fractions (Schlinger and Arnold, 1992). However, a closer look at aromatase expression in synapses of juvenile songbirds may reveal possible sex differences in local delivery of E\textsubscript{2} during development.

### 1.3.3 Dual Effect of Genes and Steroids

While both of the above two proposed alternatives to the classic model of sexual differentiation have support, it is possible that an interaction between the two mechanisms contributes to sexual differentiation alongside classic mechanisms. However, there is little research addressing how genes and local steroid production may interact. An example of this interaction is shown in zebra finches, in which the expression of 17β-HSD (Type IV), which mainly converts E\textsubscript{2} into estrone (reviewed in Compagnone and Mellon, 2000), is Z-linked (London et al., 2005). Consequently, males have greater brain 17β-HSD IV mRNA levels during development (P1-5) and adulthood than females.

### 1.4 Conclusions

Alternative mechanisms of sexual differentiation may have important implications on the study of sex differences that develop independently of gonadal hormones. Currently, the role of these alternative mechanisms in the development of sex differences is poorly understood. Songbirds may be an excellent animal model to explore the contribution of alternative mechanisms of sexual differentiation since pronounced sex differences in the song system seem to develop independently of gonadal hormones.
1.5 References


Ellegren, H., et al., 2007. Faced with inequality: chicken do not have a general dosage compensation of sex-linked genes. Bmc Biology. 5.


2. Dehydroepiandrosterone and 17beta-Estradiol in Plasma and Brain of Developing and Adult Zebra Finches

2.1 Introduction

Pronounced differences between males and females in morphology and behavior exist across vertebrate species. For instance, in many species of songbirds, males have more colorful plumage, more complex song behavior, and larger song control nuclei than females (reviewed in Brenowitz and Beecher, 2005). These sex differences are particularly pronounced in zebra finches (*Taeniopygia guttata*). The current dogma of sexual differentiation, championed by Jost (1970) and Phoenix and colleagues (1959), postulates that genes trigger gonadal development and gonadal steroids then drive sex differences in morphology and behavior. However, sexual differentiation in songbirds is not explained by this model.

Hormone manipulations in songbirds show that E2 administration to female hatchlings, followed by, T administration in adulthood, leads to masculinization of song behavior and song nuclei (Gurney, 1981; Gurney and Konishi, 1980; Simpson and Vicario, 1991a; Simpson and Vicario, 1991b). However, later studies that attempted to block estrogen receptors (ERs) with antagonists (Mathews and Arnold, 1990; Mathews and Arnold, 1991; Mathews et al., 1988) and block E2 synthesis with aromatase inhibitors (Wade and Arnold, 1996; Wade et al., 1996) in male hatchlings, were unable to prevent masculinization of their song system. Additionally, the source of E2 in males during development is unclear. Several studies have examined circulating levels of sex steroids in zebra finches and wild songbirds with conflicting results. An early study by Hutchison et al. (1984) in zebra finches found significantly higher E2 levels in male jugular
plasma at post-hatch day 3 (P3; P0 is defined as day of hatch). Subsequent studies in developing zebra finches that sampled brachial and cardiac plasma did not replicate this finding (Adkins-Regan et al., 1990; Schlinger and Arnold, 1992), but one study found higher brachial E2 levels in males at P12 (Adkins-Regan et al., 1990). The different sources of blood may partially explain inconsistencies among studies since brachial and cardiac plasma reflect systemic steroid levels, whereas jugular plasma is enriched with neutrally-synthesized steroids (Schlinger and Arnold, 1993). Interestingly, a recent study comparing brachial and jugular steroid levels across development in a wild songbird species, European starlings (Sturnus vulgaris), suggests that there is more E2 release by the brain at P4 and P6 in males than females, whereas there is more E2 uptake by the brain at P8 in females than males. (Chin et al., 2008)

Contrary to the current model of sexual differentiation, there is little evidence to suggest that gonads act as a primary source of sex steroids in developing songbirds. For example, gonadectomizing male hatchlings (Adkins-Regan and Ascenzi, 1990) and inducing functional testicular tissue in female hatchlings (Wade and Arnold, 1996) have no effect on the development of the song system. Moreover, an in vitro study of zebra finches found that female P25 brain slices co-cultured with age-matched male brain slices grew masculinized projections between the HVC and RA, two important song nuclei, and this masculinization was blocked with an ER antagonist or an aromatase inhibitor (Holloway and Clayton, 2001). Importantly, the steroidogenic enzymes for synthesizing sex steroids are expressed in the brain of developing songbirds (Jacobs et al., 1999; London et al., 2003; London et al., 2006). These studies suggest that neurosteroids, steroids synthesized de novo in the brain, may regulate brain sexual differentiation in songbirds.
While the role of $E_2$ is well-established in sexually dimorphic systems including the song system in songbirds, the role of sex steroid precursors like dehydroepiandrosterone (DHEA), which can be metabolized into T and $E_2$, is poorly understood. Since DHEA has no known nuclear receptors (reviewed in Compagnone and Mellon, 2000), it is likely that DHEA acts indirectly via intracrinology, or local metabolism in target tissues. Interestingly, Migues and colleagues (2002) found higher DHEA levels in the forebrain of female P1 chickens relative to males. Also, DHEA levels in the forebrain were ~30X higher than plasma levels. In developing European starlings, systemic DHEA levels are higher in males at P4, which falls within the likely critical period (Chin et al., 2008). Comparing brachial and jugular DHEA levels suggests that there is more DHEA release by the brain at P0 and P10 in females relative to males, whereas there is more DHEA uptake by the brain at P4 in males relative to females. While looking at steroid levels in plasma is important, it is also useful to directly measure steroid levels in the brain (Newman et al., 2008a).

In the current study, we wanted to test whether neurosteroids (‘neurosteroid hypothesis’) or local metabolism of DHEA (‘intracrinology hypothesis’) play a role in sexual differentiation of songbirds. To do so, we measured DHEA and $E_2$ levels in brachial and jugular plasma at several important ages in developing and adult male and female zebra finches. Also, we measured DHEA and $E_2$ levels in several brain regions as well as adrenals, gonads, and liver. The neurosteroid hypothesis predicts a sex difference in $E_2$ levels in the brain and jugular plasma (M>F) but not brachial plasma or peripheral tissues. Also, it predicts a possible sex difference in DHEA levels in the brain and jugular plasma (M>F) but not brachial plasma or peripheral tissues. In contrast, the intracrinology hypothesis predicts a sex difference in DHEA levels in brachial plasma and peripheral tissues (M>F) as well as brain regions (F>M). It also predicts a
possible sex difference, due to local DHEA metabolism, in E$_2$ levels in the brain and jugular plasma (M>F) and a lack of sex difference in E$_2$ brachial levels.

2.2 Materials & Methods

2.2.1 Subjects

Animals were obtained from a breeding colony at University of British Columbia (animal care permit A06-0408). Animal use followed guidelines of the Canadian Council on Animal Care. Animals were housed as previously described (Schmidt and Soma, 2008). Briefly, breeding pairs of adult zebra finches were housed separately and given millet seeds, grit, cuttlefish bone, and water ad libitum. Breeding pairs were also given a daily food supplement consisting of boiled chicken eggs, cornmeal, and bread. The light cycle provided 14 hours of light and ten hours of dark (lights on at 8:00 AM). Temperature was held constant at 23°C and relative humidity at ~50%. All nest boxes were monitored daily to record hatch date and hatchlings were marked with non-toxic colored markers to discriminate among hatchlings in a nest box.

Plasma was collected from P0, P1, P3, P4, P6, P12, P30, and adult (P90+) zebra finches and tissue was collected from P0, P1, P3, P4, P30, and P90+ zebra finches. These ages were selected based on previous reports of sex differences at these ages (Adkins-Regan et al., 1990; Casto et al., unpublished data; Chin et al., 2008; Holloway and Clayton, 2001; Hutchison et al., 1984). Tissue samples were collected from the ages thought to be most critical in development. Additionally, the ages P0 and P1 were combined, as were the ages P3 and P4, due to small plasma volumes and tissue weights at these ages. All adult subjects were sexually inexperienced and group-housed with animals of the same sex.
2.2.2 Blood Sampling

Blood was sampled as previously described (Chin et al., 2008; Sheldon et al., in press) between 9:30 and 11:30 AM from the brachial or jugular vein. Plasma samples from a given age and sex were pooled together to increase the size of individual plasma pools (Table 1). Brachial blood was collected in heparinized capillary tubes after puncturing the left brachial vein with a 30-gauge (hatchlings younger than P6) or 26-gauge (hatchlings P6 or older) needle. Jugular blood was collected in heparinized capillary tubes after puncturing the jugular vein with a 30-gauge needle (hatchlings younger than P6) or in heparinized 28.5-gauge insulin syringe (hatchlings P6 or older). Blood was collected within five min of disturbance (3.61 ± 0.05 min) and animals were sacrificed immediately after blood collection to minimize the effects of stress (Soma et al., 2004). Hatchlings were not bled a second time for at least two weeks after the first time, and never bled more than twice. Plasma was obtained by centrifugation of blood and stored at -20ºC until assayed for steroid content. Following blood collection, body measurements including body mass, wing cord length, and tarsus length were recorded.

2.2.3 Tissue Dissection

Brain tissue and peripheral tissue were dissected from male and female zebra finches. Animals were sacrificed by rapid decapitation within five min to minimize the effects of stress. The brain was chilled at -20 ºC for approximately one min prior to dissection. The brain was divided into left and right telencephalon (TEL), diencephalon (DIEN), and optic lobes (OL). Due to size constraints, only the telencephalon of hatchlings P3 and older were further separated into rostral (rTEL), caudal (cTEL), and dorsal (dTEL) sections. The dTEL contains the song nuclei HVC, which is involved in song production (reviewed in Gahr and Daisuke, 2007), and NCM,
which is involved in auditory processing, as well as the hippocampus. The rTEL contains the song nuclei mMAN, lMAN, and Area X, which are important for song learning. The cTEL contains the song nucleus RA, which is involved in song production. To our knowledge, no sex differences have been reported in the OL, and thus we did not expect sex differences in steroid levels in this region.

To dissect out the dTEL, cuts were made on the telencephalon halfway between rostral and caudal edges, halfway between dorsal and ventral edges, and halfway between the midline and lateral edges. After the dTEL was collected, tissue from the rostral edge to halfway between the rostral and caudal edges was considered rTEL and collected. Next, tissue from halfway between the rostral and caudal edges to the caudal edge of the brain was considered cTEL and collected. Left and right rTEL and cTEL were collected separately. The DIEN was located ventral to the telencephalon and collected next. After the DIEN was collected, the left and right OL, which were located lateral to the DIEN, were collected together.

The body was kept at 4ºC while the brain was dissected. To dissect the body, part of right lobe of the liver was collected. Next, the gonads were collected and lastly, both adrenals were collected. All tissue was stored at -80ºC until assayed for steroid content.

2.2.4 Steroid Extraction

Steroids were extracted from plasma and tissue using solid phase extraction with C18 columns (6 mL capacity, 500 mg, endcapped; United Chemical Technologies UCTCEC18156) as previously described (Newman et al., 2008a). Plasma pools (25 µL for P0/P1 hatchlings and 66 µL for all other ages) were prepared for extraction by bringing the samples up to 10 mL with deionized water (diH₂O). Using a vacuum manifold, C18 columns were primed with 3 mL
HPLC-grade ethanol, followed by 10 mL diH$_2$O. Next, the 10 mL sample volume was added and subsequently washed with 10 mL diH$_2$O. Lastly, steroids were eluted with 5 mL 90% HPLC-grade methanol into 7 mL borosilicate glass scintillation vials. The samples were then dried down in a SpeedVac Concentrator (Thermo Electron Corp., Savant SPD111V) at 40°C and stored at -20°C until resuspension.

For tissue samples, tissues were weighed and transferred to 13×100 mm borosilicate glass test tubes. Ice-cold diH$_2$O was added (3× tissue weight) prior to homogenization of tissue on ice with a homogenizer (Fisher Scientific, Power Gen 35). Immediately afterwards, HPLC-grade methanol was added (4× tissue weight and volume of diH$_2$O). Subsequently, samples were sonicated for 15 min (Fisher Scientific FS-20), shaken for 60 min (IKA Vibrax VXR basic), and stored at 4°C overnight. The following day, samples were shaken for 90 min, and then centrifuged at 3000g for 10 min at 2°C. The supernatant was transferred to 16×100 mm borosilicate glass test tubes. In cases where the supernatant volume was greater than 1 mL, only 1 mL of supernatant was transferred. As with plasma samples, tissue sample supernatants were brought up to 10 mL with diH$_2$O, loaded onto primed and equilibrated C18 columns, washed with diH$_2$O, eluted with 90% HPLC-grade methanol, dried down, and stored at -20°C.

2.2.5 Steroid Resuspension

Prior to measuring steroids, samples were resuspended in phosphate buffered saline with gelatin (PBSG). Absolute ethanol (1.5% final concentration) was added to the vials and briefly vortexed to bring steroids into solution (Newman et al., 2008a). PBSG was then added to bring samples up to final volume. Plasma samples from P12 or younger birds were resuspended in 250 µL and assayed as singletons, whereas plasma samples from P30 or older birds were resuspended
in 500 µL and assayed in duplicate. Brain samples from P4 and younger birds were resuspended in 125 µL, pooled from two individuals of the same age and sex, and assayed in singleton. Brain samples from P30 and older birds were resuspended in 500 µL and assayed in duplicate. These volumes were determined from prior validations (Newman et al., 2008a) and steroid levels in developing European starlings (Chin et al., 2008). Resuspended samples were shaken at 1500 rpm for 60 min (IKA Vibrax VXR basic) and stored at 4ºC overnight. On the following day, samples were again shaken at 1500 rpm for 60 min, prior to radioimmunoassay (RIAs).

To quantify recovery of DHEA and E₂, pools of plasma and brain tissue were spiked with 194 pg of DHEA and 6 pg of E₂ and then compared to otherwise identical unspiked pools. Recovery was calculated for both resuspension volumes (250 and 500 µL). Recovery of DHEA from plasma was 84.47 ± 7.05% (n=4 pairs, 250 µL) and 59.69 ± 5.39% (n=4 pairs, 500 µL). Recovery of DHEA from brain tissue was 61.01 ± 4.81% (n=7 pairs, 250 µL) and 51.95 ± 4.27% (n=4 pairs, 500 µL). Recovery of E₂ from plasma was 52.95 ± 4.26% (n=7 pairs, 250 µL) and 55.31 ± 3.12% (n=7 pairs, 500 µL). Recovery of E₂ from brain tissue was 12.64 ± 5.46% (n=7 pairs, 250 µL) and 4.96 ± 0.88% (n=7 pairs, 500 µL).

2.2.6 RIAs

DHEA was measured using a double antibody RIA that uses ¹²⁵I-DHEA as the tracer (Diagnostic Systems Laboratories, DSL-8900), as previously described (Chin et al., 2008). The DHEA assay was modified as per Granger et al. (1999), and has been previously used with songbirds (Chin et al., 2008; Goodson et al., 2005; Newman et al., 2008b). Briefly, using PBSG, DHEA kit standards were diluted 10×, the primary antibody was diluted 4×, and the tracer was diluted (between 1 and 4×) depending on its age. The DHEA antibody has a low cross-reactivity
with DHEA-S (0.02%), 16β-OH DHEA (0.041%), AE (0.46%), T (0.028%), and E2 (<0.004%; E. Chin, unpublished data). Assay sensitivity was 2 pg/tube and sample volume was 100 µL. The addition of ethanol to resuspend samples did not affect this DHEA assay (Newman et al., 2008a). Interassay variation was 13.65% and intraassay variation was 6.36%. Three unextracted controls (3, 15, and 150 pg) were run with each assay to determine accuracy. For the 3 pg control, an average measurement of 2.55 ± 0.17 pg (n=10) was obtained; for the 15 pg control, an average measurement of 13.75 ± 0.41 pg (n=10) was obtained; and for the 150 pg control, an average measurement 152.49 ± 4.53 pg (n=10) was obtained. All extracted water blanks (n=6) were below the lowest standard (<2 pg DHEA).

E2 was measured using a double antibody RIA that uses 125I-E2 as the tracer (Diagnostic Systems Laboratories, DSL-4800). This kit was also modified to increase sensitivity (Shah et al., unpublished data). Using PBSG, E2 standards were diluted between 5.3× and 66.7×, the primary antibody was diluted 3.5×, and the tracer was diluted 3×. The E2 antibody has a low cross-reactivity with estrone (2.4%), estrone-sulfate (0.01%), estrone-3-glucuronide (<0.01%), estriol (0.64%), 17α-estradiol (0.21%), 17β-estradiol-3-glucuronide (2.56%), T (<0.01%), and DHEA (<0.01%). Assay sensitivity was 0.1875 pg E2/tube and sample volume was 300 µL (200 µL of PBSG was added to 100 µL of sample). The final ethanol concentration was therefore 0.5%, which did not affect this assay (Fig. 2). Interassay variation was 12.54% and intraassay variability was 7.80%. Three unextracted controls (0.3, 0.5, and 1.5 pg) were run with each assay to determine accuracy. For the 0.3 pg control, an average measurement of 0.33 ± 0.02 pg (n=9) was obtained; for the 0.5 pg control, an average measurement of 0.53 ± 0.03 pg (n=9) was obtained; and for the 1.5 pg control, an average measurement 1.62 ± 0.04 pg (n=9) was obtained.
Three of the extracted water blanks (n=9) were below the lowest standard (<0.1875 pg E₂). The detection limit was thus set at the average + 2 SD of the water blanks (0.240 pg).

### 2.2.7 Sex Genotyping

Subsequent to blood sampling, some blood was dabbed onto filter paper and stored at -20°C. Hatchlings were genotyped by polymerase chain reaction (PCR) as previously described (Griffiths et al., 1996; Love et al., 2005). Briefly, DNA was isolated from the red blood cell samples using Insta-gene matrix (Bio-Rad Laboratories, Hercules, California, Cat. No. 732-6030) following the manufacturer’s protocol. DNA concentration was quantified with a spectrophotometer (Beckman Coulter DU 640). PCR amplification was run using the P2 (5’-TCTGCATCGCTAAATCCTTT) and CW (5’-AGAAATCATTCCAGAAGTTCA) primer set. PCR products were separated on a 3% agarose gel with ethidium bromide (70V for 70 min) and visualized with a UV transilluminator. Adult males and females (n=4 of each sex) were used as positive controls.

### 2.2.8 Statistics

Two-way ANOVAs were used to assess the effects of age and sex on each of the three body measurements and also to assess the effects of ethanol concentration and E₂ dose on measured E₂. For plasma, three-way ANOVAs were used to assess the effects of age, sex, and site of blood sampling on plasma DHEA and E₂ levels. Also, a two-way ANOVA was used to assess the effects of age and sex on jugular minus brachial “difference scores” for plasma DHEA and E₂ samples. For brain tissue samples, P0/P1 samples were analyzed separately since fewer regions were collected at this age, and the remaining ages were analyzed together. A two-way ANOVA was used to assess the effects of sex and tissue type on brain DHEA and E₂ levels in at
P0/P1 and a three-way ANOVA was used to assess the effects of age, sex, and tissue type on brain DHEA and E2 levels at the remaining ages. Lastly, a three-way ANOVA was used to assess the effects of age, sex, and tissue type on peripheral tissue DHEA and E2 levels. Data was log transformed when appropriate. Post-hoc ANOVA analysis was used to assess relevant interactions and sex differences. Significant main effects of age, blood sampling site, or tissue type were not further analyzed.

Any samples with values below the lowest standard on the standard curve were set to zero. Also, all samples were corrected for recovery. All statistical analysis was done on SPSS 15.0 and statistical significance was set at \( p = 0.05 \).

2.3 Results

2.3.1 Body Measurements

Body mass (Fig. 1), wing cord length (data not shown), and tarsus length (data not shown) were measured at all ages to assess physical development in males and females. There were significant correlations between body mass and wing cord \( (r=0.964, p<0.001) \), body mass and tarsus length \( (r=0.970, p<0.001) \), and wing cord and tarsus length \( (r=0.918, p<0.001) \).

Two-way ANOVAs were used to analyze the effects of age and sex on body mass, wing cord length, and tarsus length. A significant main effect of age was found on body mass \( (F_{5,374}=2557.013; p<0.001) \), wing cord length \( (F_{5,358}=5385.900; p<0.001) \), and tarsus length \( (F_{5,358}=1517.245; p<0.001) \). Also, there was a significant main effect of sex on body mass \( (F_{1,374}=6.983, p=0.009) \) and wing cord length \( (F_{1,358}=5.619, p=0.018) \), as well as a significant interaction between age and sex for body mass \( (F_{5,374}=3.699, p=0.003) \). Post hoc ANOVA
analysis of the effect of sex at each age revealed a significant sex difference in body mass at P6 (F>M; $F_{1,374}=20.23, p<0.001$).

2.3.2 Assay Validations

The effect of ethanol (to resuspend steroids) on the $E_2$ assay was examined by measuring $E_2$ in samples with known amounts of $E_2$ (0.3, 0.5, or 1.5 pg) and known concentrations of ethanol (0, 0.5, or 1%; Fig. 2). In a two-way ANOVA, there was no effect of ethanol concentration and no interaction between $E_2$ dose and ethanol concentration.

2.3.3 Plasma DHEA and $E_2$ Levels

DHEA levels were similar between P0/P1 and P12, peaked at P30, and decreased by P90+ in both brachial (Fig. 3A) and jugular (Fig. 3B) plasma. DHEA was detectable in 56% of brachial plasma samples and 66% of jugular plasma samples (Table 2A). A three-way ANOVA was used to determine the effects of age, sex, and site of blood sampling on plasma DHEA levels. There was a significant main effect of age ($F_{5,119}=16.652, p<0.001$), but not of sex or site of blood sampling. No significant interactions were found.

To compare DHEA levels in brachial and jugular plasma, the average brachial DHEA at any given age and sex was subtracted from individual jugular DHEA levels (Fig. 4), to yield difference scores as previously described (Chin et al., 2008). Difference scores greater than zero may indicate higher DHEA uptake by the brain and differences scores lower that zero may indicate higher DHEA release by the brain. A two-way ANOVA was used to determine the effect of age and sex on difference scores. A significant main effect of age ($F_{5,63}=7.483, p<0.001$) but not sex was found. No significant interactions were found.
E2 levels increased from P0/P1 to P6, then decreased to P30, and peaked at P90+ in both brachial (Fig. 5A) and jugular plasma (Fig. 5B). Similar to DHEA, E2 was detectable in 61% of brachial plasma samples and 63% of jugular plasma samples (Table 2B). For E2, the three-way ANOVA showed a significant main effect of age ($F_{5,119}=16.547$, $p<0.001$) but not of sex or site of blood sampling. No significant interactions were found. An analysis of jugular minus brachial E2 difference scores (Fig. 6) with a two-way ANOVA did not show any significant main effects or a significant interaction.

2.3.4 Brain DHEA and E2 Levels

DHEA levels were measured in the TEL, DIEN, and OL at P0/P1 (Fig. 7A) and cTEL, dTEL, rTEL, DIEN, and OL at P3/P4 (Fig. 7B), P30 (Fig. 7C) and P90+ (Fig. 7D). A two-way ANOVA was used to determine the effects of age and tissue type on brain DHEA levels at P0/P1. A significant main effect of sex (M>F; $F_{1,23}=4.417$, $p=0.047$) and tissue type ($F_{2,23}=3.789$, $p=0.038$) was found. No significant interaction was found. A three-way ANOVA was used to determine the effects of age, sex, and tissue type on brain DHEA concentration at the remaining ages. A significant main effect of age ($F_{2,161}=15.235$, $p<0.001$) and a significant interaction between sex and tissue type ($F_{4,161}=3.07$, $p=0.018$) were found. Post hoc ANOVA analysis of the effect of sex at each tissue type and age, showed a significant sex difference in rTEL at P30 (F>M; $F_{1,161}=17.68$, $p<0.001$).

Similarly, E2 levels were measured in the TEL, DIEN, and OL at P0/P1 (Fig. 8A) and cTEL, dTEL, rTEL, DIEN, and OL at P3/P4 (Fig. 8B), P30 (Fig. 8C) and P90+ (Fig. 8D). The two-way ANOVA showed no significant main effects or interactions at P0/P1. The three-way ANOVA showed a significant main effect of age ($F_{2,157}=5.765$, $p=0.004$) and tissue type.
(F_{4,157}=3.237, p=0.014) on brain E_2 levels at the remaining ages. No significant interactions were found.

2.3.5 Peripheral Tissue DHEA and E_2 Levels

DHEA levels were measured in adrenals, gonads, and liver tissue at P0/P1 (Fig. 9A), P3/P4 (Fig. 9B), P30 (Fig. 9C), and P90+ (Fig. 9D). A three-way ANOVA was used to determine the effects of age, sex, and tissue type on peripheral tissue DHEA levels. A significant main effect of age was found (F_{3,119}=13.241, p<0.001) and tissue type (F_{2,119}=27.004, p<0.001) were found. A significant interaction between age and tissue type (F_{6,119}=2.850, p=0.013) was also found. Post hoc ANOVA analysis looking at the effect of sex at each tissue type and age showed no significant sex differences.

Similarly, E_2 levels were measured in adrenals, gonads, and liver tissue at P0/P1 (Fig. 10A), P3/P4 (Fig. 10B), P30 (Fig. 10C), and P90+ (Fig. 10D). For E_2, the three-way ANOVA showed a significant main effect of age (F_{3,118}=7.412, p<0.001) and tissue type (F_{2,118}=4.613, p=0.012), as well as a significant interaction between age and tissue type (F_{6,118}=4.112, p=0.001), on peripheral tissue E_2 levels. Post hoc ANOVA analysis looking at the effect of sex at each tissue type and age showed a significant sex difference in gonads at P90+ (F>M; F_{1,182}=5.17, p=0.025).

2.4 Discussion

To our knowledge, this is the first study to compare brachial and jugular levels of DHEA and E_2 in developing and adult zebra finches and the first to measure in vivo levels of DHEA and E_2 in brain and peripheral tissue of developing and adult zebra finches.
2.4.1 Plasma DHEA and E2 Levels

In males and females, plasma DHEA and E2 levels followed similar trends at all measured ages. Surprisingly, we were not able to replicate any of the sex differences found by other researchers in jugular E2 at P3 (Hutchison et al., 1984) and brachial E2 at P12 (Adkins-Regan et al., 1990). Interestingly, DHEA levels in plasma peaked at P30, whereas E2 levels in plasma peaked at P6 and again at P90+. Whereas brachial DHEA peaked at P30 in zebra finches, it peaked earlier in development at P4 in European starlings (Chin et al., 2008), indicating a clear species difference within songbirds. Also, E2 levels were mostly non-detectable across development in European starlings but mostly detectable in zebra finches, especially at older ages.

2.4.2 Brain DHEA and E2 Levels

Brain DHEA levels were slightly higher in males than females in some brain regions at P0/P1 and P3/P4, significantly higher in females than males in some brain regions at P30, and similar between males and females at P90+. Females had higher DHEA levels in rTEL at P30 than males but no other significant sex differences were found. Higher DHEA levels in rTEL of P30 females relative to males, contrasts with higher DHEA in jugular plasma found in males relative to females. A possible explanation is that jugular plasma is indicative of steroid levels in the whole brain and sex differences in individual brain regions are diluted in jugular plasma. Future studies need to further evaluate whether jugular plasma is a good correlate of brain steroid levels. Also, no sex differences in DHEA levels were found in any brain regions at P90+ suggesting that sex differences in DHEA levels at P30 are possibly involved in sexual differentiation of the song system. The jugular minus brachial difference scores and the fact that
DHEA levels in rTEL of P30 females are higher than brachial DHEA levels at that age and higher than DHEA levels in other brain regions at that age, suggest that the sex difference in rTEL at P30 (F>M) is due to higher DHEA release by the brain in females relative to males.

Interestingly, males had more brain samples detectable for DHEA than females at P0/P1 (42% for males versus 13% for females) and at P3/P4 (43% for males versus 22% for females) but brain samples were similarly detectable between males and females at the older ages. This corresponds with a trend seen in many brain regions of higher DHEA levels in males relative to females at P0/P1 and P3/P4. Taken together, this suggests that DHEA uptake by the brain may be higher in females than males or that DHEA release by the brain may be higher in males than females at P0/P1 and P3/P4.

The observed results provide some evidence that DHEA may contribute to sexual differentiation. It is unlikely that DHEA acts directly via nuclear receptors in the brain to trigger sexual differentiation since it has no known receptor and only binds very weakly to ARs (Chen et al., 2005; Shi-Fang Lu, 2003). However, it has been shown to modulate activity of nongenomic receptors like GABA_A, NMDA, and sigma receptors (reviewed in Compagnone and Mellon, 2000), and may act directly on these receptors to affect sexual differentiation. It is also possible that DHEA is locally metabolized in the brain to another sex steroid, which then influences sexual differentiation.

Brain E_2 levels were similar between males and females and similarly detectable in males and females at all ages. This contradicts with previous findings of higher E_2 synthesis in cultured brain slices of males relative to females at P30 (Holloway and Clayton, 2001). However, in vitro E_2 synthesis may not accurately reflect in vivo functioning. Relative to plasma E_2 levels, brain E_2
levels were generally higher (~2-3x), which may be a reflection of differing steroid recoveries from plasma and brain, lower metabolism in brain relative to plasma, or higher accumulation/synthesis in brain relative to plasma.

The observed results provide little evidence for E2 contributing to sexual differentiation in songbirds via de novo synthesis or by being locally metabolized in the brain. However, it is possible that E2 synthesis is more localized at the synaptic level and that smaller brain tissue dissections are required to be able to detect sex differences in E2 levels. Alternatively, it is possible that E2 is rapidly metabolized into another estrogen.

2.4.3 Peripheral Tissue DHEA and E2 Levels

DHEA and E2 were measured in peripheral tissues to determine other potential sources of these sex steroids during development. DHEA levels in adrenals and gonads were highly variable in developing zebra finches, possibly due to the small size of these organs during early development (~0.1 to 2 mg) that may have led to problems in dissecting and accurately weighing these tissue samples. While absolute DHEA levels in adrenals and gonads highly fluctuated across development, relative levels between males and females remained generally similar and generally much higher than plasma levels, suggesting that these tissues act as a systemic source of DHEA during development and in adulthood. Interestingly, DHEA levels in liver were lower relative to gonads and adrenals, but several fold higher relative to plasma and brain levels indicating that liver tissue may also synthesize DHEA, which has been previously suggested in rodents (Katagiri et al., 1998; Vianello et al., 1997).

Similarly, E2 levels in gonads and adrenals were generally several fold higher than plasma levels suggesting that these tissues may act as sources of E2 across development. Also, E2
levels in these tissues during early development were highly variable, again possibly due to the small size of these organs. Interestingly, none of the gonadal or adrenal samples had detectable E$_2$ at P30, which may be due to decreased synthesis or increased metabolism at this age. Correspondingly, brachial E$_2$ levels were lower at P30 relative to the earlier ages of P6 and P12. E$_2$ levels in liver tissue were several fold higher relative to plasma levels but comparable to brain levels. Not surprisingly, females had higher E$_2$ levels than males in gonads at P90+.

2.5 Conclusions

The current study finds little evidence to indicate that gonadal hormones are involved in sexual differentiation of songbirds and further supports the role of alternative mechanisms of sexual differentiation in songbirds. There was no evidence of sex differences in systemic DHEA levels, suggesting that the intracrinology hypothesis does not explain sexual differentiation in songbirds. However, there was a sex difference found in local DHEA levels in the brain at P30, suggesting that the neurosteroid hypothesis may partly explain sexual differentiation in songbirds. However, further research needs to determine whether higher DHEA levels in rTel at P30 in females relative to males was due to higher synthesis in females or higher metabolism in males. There was little indication from this study that local metabolism of DHEA to E$_2$ or de novo synthesis of E$_2$ contributes to sexual differentiation of the song system. However, future studies need to address whether other androgens or estrogens play a primary role in sexual differentiation of the song system.
Table 1. Sample size for DHEA and E$_2$ plasma samples. The number of animals pooled to obtain the sample sizes is recorded in brackets.

<table>
<thead>
<tr>
<th>Age</th>
<th>Males Brachial</th>
<th>Males Jugular</th>
<th>Females Brachial</th>
<th>Females Jugular</th>
</tr>
</thead>
<tbody>
<tr>
<td>P0/P1</td>
<td>4 (14)</td>
<td>6 (14)</td>
<td>5 (12)</td>
<td>4 (9)</td>
</tr>
<tr>
<td>P3/P4</td>
<td>5 (14)</td>
<td>6 (13)</td>
<td>5 (18)</td>
<td>4 (8)</td>
</tr>
<tr>
<td>P6</td>
<td>5 (9)</td>
<td>7 (5)</td>
<td>6 (9)</td>
<td>6 (4)</td>
</tr>
<tr>
<td>P12</td>
<td>6 (5)</td>
<td>9 (7)</td>
<td>6 (7)</td>
<td>7 (7)</td>
</tr>
<tr>
<td>P30</td>
<td>7 (9)</td>
<td>4 (4)</td>
<td>5 (7)</td>
<td>8 (8)</td>
</tr>
<tr>
<td>P90+</td>
<td>7 (10)</td>
<td>7 (5)</td>
<td>7 (10)</td>
<td>7 (5)</td>
</tr>
</tbody>
</table>
Table 2. Percent of detectable samples for (A) DHEA and (B) E\textsubscript{2} plasma samples.

(A)

<table>
<thead>
<tr>
<th>Age</th>
<th>DHEA Males</th>
<th></th>
<th>Females</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Brachial</td>
<td>Jugular</td>
<td>Brachial</td>
</tr>
<tr>
<td>P0/P1</td>
<td>50</td>
<td>50</td>
<td>0</td>
</tr>
<tr>
<td>P3/P4</td>
<td>40</td>
<td>67</td>
<td>40</td>
</tr>
<tr>
<td>P6</td>
<td>40</td>
<td>14</td>
<td>33</td>
</tr>
<tr>
<td>P12</td>
<td>67</td>
<td>78</td>
<td>33</td>
</tr>
<tr>
<td>P30</td>
<td>100</td>
<td>100</td>
<td>80</td>
</tr>
<tr>
<td>P90+</td>
<td>86</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>

(B)

<table>
<thead>
<tr>
<th>Age</th>
<th>E\textsubscript{2} Males</th>
<th></th>
<th>Females</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Brachial</td>
<td>Jugular</td>
<td>Brachial</td>
</tr>
<tr>
<td>P0/P1</td>
<td>0</td>
<td>33</td>
<td>0</td>
</tr>
<tr>
<td>P3/P4</td>
<td>80</td>
<td>50</td>
<td>60</td>
</tr>
<tr>
<td>P6</td>
<td>60</td>
<td>86</td>
<td>83</td>
</tr>
<tr>
<td>P12</td>
<td>83</td>
<td>56</td>
<td>100</td>
</tr>
<tr>
<td>P30</td>
<td>43</td>
<td>75</td>
<td>20</td>
</tr>
<tr>
<td>P90+</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>
Table 3. Sample sizes for (A) DHEA and (B) E₂ brain samples. All samples at P0/P1 and P3/P4 were pools of two animals of the same age and sex.

(A)

<table>
<thead>
<tr>
<th>Age</th>
<th>Males</th>
<th>Females</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TEL</td>
<td>rTEL</td>
</tr>
<tr>
<td>P0/P1</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>P3/P4</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>P30</td>
<td>7</td>
<td>7</td>
</tr>
<tr>
<td>P90+</td>
<td>7</td>
<td>7</td>
</tr>
</tbody>
</table>

(B)

<table>
<thead>
<tr>
<th>Age</th>
<th>Males</th>
<th>Females</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TEL</td>
<td>rTEL</td>
</tr>
<tr>
<td>P0/P1</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>P3/P4</td>
<td>4</td>
<td>5</td>
</tr>
<tr>
<td>P30</td>
<td>7</td>
<td>7</td>
</tr>
<tr>
<td>P90+</td>
<td>7</td>
<td>7</td>
</tr>
</tbody>
</table>
Table 4. Sample sizes for (A) DHEA and (B) E2 peripheral tissue samples. All samples at P0/P1 and P3/P4 were pools of two animals of the same age and sex.

(A)

<table>
<thead>
<tr>
<th>Age</th>
<th>Males</th>
<th></th>
<th>Females</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Gonads</td>
<td>Adrenals</td>
<td>Liver</td>
<td>Gonads</td>
</tr>
<tr>
<td>P0/P1</td>
<td>6</td>
<td>6</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>P3/P4</td>
<td>4</td>
<td>5</td>
<td>5</td>
<td>6</td>
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<tr>
<td>P30</td>
<td>7</td>
<td>7</td>
<td>7</td>
<td>7</td>
</tr>
<tr>
<td>Adult</td>
<td>6</td>
<td>7</td>
<td>7</td>
<td>6</td>
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</tbody>
</table>

(B)

<table>
<thead>
<tr>
<th>Age</th>
<th>Males</th>
<th></th>
<th>Females</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Gonads</td>
<td>Adrenals</td>
<td>Liver</td>
<td>Gonads</td>
</tr>
<tr>
<td>P0/P1</td>
<td>6</td>
<td>5</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>P3/P4</td>
<td>4</td>
<td>4</td>
<td>7</td>
<td>5</td>
</tr>
<tr>
<td>P30</td>
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<tr>
<td>Adult</td>
<td>7</td>
<td>7</td>
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<td>6</td>
</tr>
</tbody>
</table>
Figure 1. Changes in body mass during development in zebra finches. Data are shown as means ± SEM.
Figure 2. Effect of absolute ethanol concentration (0, 0.5, or 1%) on measured E₂ at 0.3, 0.5, or 1.5 pg of added, or spiked, E₂. Data are shown as means ± SEM.
Figure 3. DHEA levels in (A) brachial and (B) jugular plasma across development. Data are shown as means ± SEM.

(A)                                                                                     (B)

![Graph A: Brachial DHEA levels across development.](image)

Males

Females

![Graph B: Jugular DHEA levels across development.](image)

Age

P0/P1 P3/P4 P6 P12 P30 P90+

Brachial DHEA (pg/mL)

Jugular DHEA (pg/mL)

0 500 1000 1500 2000 2500 3000 3500

3500 3000 2500 2000 1500 1000 500 0

0 500 1000 1500 2000 2500 3000 3500

Age
Figure 4. Jugular-brachial difference scores for plasma DHEA samples. Data are shown as means ± SEM.
Figure 5. E₂ levels in (A) brachial and (B) jugular plasma throughout development. Data are shown as means ± SEM.
Figure 6. Jugular-brachial difference scores for plasma E₂ samples. Data are shown as means ± SEM.
Figure 7. DHEA levels in the brain at (A) P0/P1, (B) P3/P4, (C) P30, and (D) P90+. Percent of detectable samples are shown along the x-axis, below the vertical bars. Data are shown as means ± SEM.
Figure 8. E₂ levels in the brain at (A) P0/P1, (B) P3/P4, (C) P30, and (D) P90+. Percent of detectable samples are shown along the x-axis, below the vertical bars. Data are shown as means ± SEM.
Figure 9. DHEA levels in peripheral tissues at (A) P0/P1, (B) P3/P4, (C) P30, and (D) P90+.

Percent of detectable samples are shown along the x-axis, below the vertical bars. Data are shown as means ± SEM.
Figure 10. E₂ levels in peripheral tissues at (A) P0/P1, (B) P3/P4, (C) P30, and (D) P90+. Percent of detectable samples are shown along the x-axis, below the vertical bars. Data are shown as means ± SEM.
2.5 References


3. General Discussion and Conclusions

The current study attempted to determine whether alternative mechanisms like neurosteroids and local metabolism of steroid precursors are involved in sexual differentiation of songbirds. The findings of this study indicate that dehydroepiandrosterone (DHEA) may be involved in sexual differentiation but surprisingly, also further challenged the idea that 17β-estradiol (E2) plays a primary role in triggering sexual differentiation of the song system. In agreement with our findings, a previous study by Chin et al. (2008) also found generally low levels of E2 and few sex differences in brachial and jugular plasma of developing European starlings, which have a less sexually dimorphic song system than zebra finches (reviewed in Schlinger, 1998). However, it is possible that E2 is being rapidly metabolized into another estrogen or perhaps, E2 synthesis is further localized at the cellular level. For instance, there is evidence of higher synaptic aromatase expression in adult male songbirds relative to females (Peterson et al., 2005) and a similar sex difference may also exist in relevant song nuclei of juvenile songbirds.

Future studies could investigate whether other androgen and estrogen metabolites are involved in sexual differentiation of the song system by incubating male and female brain tissue from developing songbirds with DHEA and measuring several androgen and estrogen metabolites via HPLC or radioimmunoassays. Also, future studies should address whether sex differences are present at other important ages during development in songbirds. It is also important to measure E2 synthesis in smaller brain regions, possibly by measuring E2 in specific micropunches of brain tissue using the Palkovits punch technique (Palkovits, 1983; Vockel et al., 1990). Alternatively, it may be possible to use immune-electron microscopy to examine aromatase expression at synapses in developing zebra finches (Peterson et al., 2005).
Additionally, future studies should also determine the effects of experimentally manipulating DHEA levels on sexual differentiation. For instance, DHEA synthesis can be inhibited by administering YM166 in Silastic implants.

In conclusion, this study further supported the idea that alternative mechanisms are involved in sexual differentiation of songbirds. While the findings of this study provided some evidence for the neurosteroid and intracrinology hypotheses, little evidence was found to support the classical mechanisms of sexual differentiation in songbirds. The alternative mechanism that genes directly influence sexual differentiation was not examined in this study and its role in songbirds needs to be further explored. Lastly, it is important to examine the role of alternative mechanisms of sexual differentiation in other animal models, including mammals, in order to fully understand the implications of these alternative mechanisms on sex differences.
3.1 References


Appendices

Appendix A

THE UNIVERSITY OF BRITISH COLUMBIA

ANIMAL CARE CERTIFICATE

Application Number: A07-0755
Investigator or Course Director: Kiran Sema
Department: Psychology, Department of

Animals:

   Birds - Other zebra finch (Taeniopygia guttata) 150

Start Date:          October 26, 2007          Approval Date: December 17, 2007

Funding Sources:
Funding Agency: Natural Sciences and Engineering Research Council of Canada (NSERC)
Funding Title: Steriods and songbird behavior

Unfunded title: N/A

The Animal Care Committee has examined and approved the use of animals for the above experimental project.

This certificate is valid for one year from the above start or approval date (whichever is later) provided there is no change in the experimental procedures. Annual review is required by the CCAC and some granting agencies.

A copy of this certificate must be displayed in your animal facility.

Office of Research Services and Administration