STEROIDS AND SEXUAL DIFFERENTIATION OF THE SONGBIRD BRAIN

by

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ABSTRACT

Sexual differentiation of the songbird brain has traditionally thought to be driven by gonadal testosterone (T) and estradiol (E_2). Recent studies have indicated that other sources of sex steroids may be responsible. Moreover, these alternative sources may release precursors, such as dehydroepiandrosterone (DHEA), which are subsequently converted downstream to sex steroids, such as E_2, only at target tissues. This thesis examines plasma and tissue hormone profiles during development and possible alternative sources of sex steroids, including the adrenals, brain and liver, during the nestling phase in the European starling (*Sturnus vulgaris*). In the first experiment, wild starling chicks were blood sampled from either the brachial wing vein or the jugular vein from hatch (P0) to fledging (P20). In the second experiment, brain and peripheral tissues were collected from wild starling chicks at P0, P6 and P8. Hormones were extracted from samples using solid phase extraction, and then measured using radioimmunassays. Plasma DHEA was higher in males than females at P4 in the brachial vein, whereas plasma DHEA was higher in females than males at P0 and P10 in the jugular vein. No sex difference was found in plasma E_2. Brain DHEA was low, but detectable, in all regions at all ages, and a sex difference was only detected at P0. Brain E_2 was non-detectable in all regions at all ages. DHEA was detectable in all peripheral tissues, with the gonadal tissue containing the highest levels of DHEA. Taken together, these data suggest that the gonads may be the primary source of circulating DHEA, and that the adrenals may be a secondary source during the nestling phase.

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CO-AUTHORSHIP STATEMENT

While the bulk of the field work and lab work for this thesis was completed by myself, E.H. Chin, the following co-authors are listed on the manuscript: Kimberly L. Schmidt, Lani D. Sheldon, Amit H. Shah, and Oliver P. Love.

Both K.L. Schmidt and L.D. Sheldon assisted in field work by collecting samples, and performing daily nestbox checks and collecting growth data. Furthermore, both individuals assisted in lab work: K.L. Schmidt assisted in the bulk of the SPE extractions for both the plasma and brain tissue samples, while L.D. Sheldon assisted in genotyping.

A.H. Shah assisted in the rest of the SPE extractions for the brain tissue and periphery tissue samples.

O.P. Love assisted in fieldwork also by collecting samples, performing daily nestbox checks, and assisting in collection of growth data.

Chapter One: Literature Review

INTRODUCTION

Sex steroids during development can have profound and long-lasting effects on brain, behaviour and physiology. During a critical or sensitive period, sex steroids can have organizational effects on the brain, and subsequently the behaviour controlled by affected brain regions. Presence or absence of sex steroids during this critical period can affect development, pushing brain and behaviour towards either a male or female direction. These organizational effects of steroids are often permanent and irreversible.

In mammals, it is well established that the gonads are the source of sex steroids during the neonatal stage, and removal of this gonadal source during development leads to a profound change in behaviour at the adult stage of life. Guinea pigs exposed to testosterone propionate (TP) in utero will show more masculine copulatory behaviours and less feminine copulatory behaviours in adulthood compared to unexposed females (Phoenix, Goy, Gerall, and Young, 1959). In birds, circulating sex steroids during development have also been examined in precocial and altricial species. Evidence supports the gonads as the source of sex steroids in precocial species such as the Japanese quail (*Coturnix japonica*) and the chicken (Ottinger, Pitts, and Abdelnabi, 2001; Sayag, Snapir, Arnon, Halawani, Grimm, and Robinzon, 1991). However, the source of sex steroids during development in altricial birds, particularly songbirds, is not clear. This review will examine developmental studies of circulating sex steroids in both mammalian and avian species, investigate the possible sources of these hormones, and how these sources differ between these species.

Mammals

Several studies have looked at sex steroid levels during development in mammals, particularly in rats, monkeys and hyenas. In rats, both brain and circulating plasma levels of steroids have been examined, but mainly in the first few hours post-partum, rather than throughout development. One study examined progesterone (PROG), testosterone (T), 5α-dihydrotestosterone (5α-DHT) and 17β-estradiol (E_2) levels in serum and the hypothalamus between embryonic day 21 (E21) to 24 hours post-partum (Rhoda, Corbier, and Roffi, 1984). There was no sex difference in serum and hypothalamic PROG. In males, serum and hypothalamic T levels surge 1-2 hours post-partum, and subsequently decline to levels seen at birth. At no point was T detectable in female serum or hypothalamus. 5α-DHT was not detectable in the serum or hypothalamus in either sex. There was no sex difference in serum E_2, but males had higher hypothalamic levels of E_2 than females throughout all the stages of life examined. Another study examined brain E_2 content in newborn rats, focusing on regional differences. Immediately post-partum, males had higher E_2 levels in the frontal cortex, hypothalamus and pre-optic area than females (Amanteau, Alt, Stamps, and McCarthy, 2004). By post-partum day 1 (P1), E_2 levels had declined such that only the hypothalamus maintained a sex difference. In another study, serum levels of 17-OH Pregnenolone (17-OH PREG), dehydroepiandrosterone (DHEA), 17-OH PROG and androstenedione (AE) were examined in rats from birth to P98. Blood was taken from the abdominal aorta. There was no sex difference in any of the steroids measured, but all steroids decreased significantly with increasing age (Tagawa, Katagiri, and Kobayashi, 2005). At no time during any of these studies did females have higher steroid levels than males.

Two studies in monkeys have examined plasma and serum concentrations of sex steroids. The first study was in male crab-eating monkeys (*Macaca fascicularis*) from birth to adulthood. Levels of DHEA, AE, 5α-DHT, and T were measured in plasma from the cephalic, basilic or saphenous vein depending on the age of the subjects. DHEA levels were high after birth, decreased at 1 year of age, and remained stable until puberty, when levels declined further (Meusy-Dessolle and Dang, 1985). AE remained constant throughout life with a slight decrease at 3 years of age, but then increased again after 4-5 years. T and 5α-DHT remained low until puberty, when they increased in parallel and concommittantly with the establishment of spermatogenesis, and continued to increase to adulthood. Another study examined levels of AE, T, and 5α-DHT in umbilical circulation of fetal Rhesus monkeys (*Macaca mulatta*) beginning at the time of gonadal differentiation. AE was higher in males than females on 79 – 84 days of gestation (Resko, Ellinwood, Pasztor, and Buhl, 1980). T was significantly higher in males than females in all gestational stages measured, and 5α-DHT was higher in males than females on 100 – 133 days of gestation as well as 140 – 163 days of gestation.

Non-passerine birds

The majority of work examining circulating sex steroids in precocial birds has been done in chickens (*Gallus domesticus*) and Japanese quail (*Coturnix japonica*). Work in these two species has been done in both pre- and post-hatch chicks, and has demonstrated that sex steroids are present in plasma in detectable levels in embryos as well as post-hatch chicks.

In chickens, plasma T levels were detectable in both male and female chicks. Male chicks had relatively high pre-hatch T levels in plasma obtained via cardiac puncture, and these levels began to increase P21 to a peak at P35 (Tanabe, Nakamura, Fujioka, and Doi, 1979). While one study reported a sex difference in plasma T from days 7 to 17 of incubation in blood collected from extraembryonic blood vessels (Woods, Simpson, and Moore, 1975), a separate study showed no sex difference in plasma T levels at embryonic ages in blood collected via cardiac puncture or venous vessels (Tanabe, Saito, and Nakamura, 1986). Meanwhile, plasma E_2 levels in females (from cardiac puncture) were 10 times lower than T levels from day 17 of embryonic development (E17) to P42, while levels in male chicks were very low or non-detectable (Tanabe et al., 1979).

Evidence in chickens suggests that gonads are the source of steroids both pre-and post-hatch (Galli and Wassermann, 1973; Guichard, Cedard., Mignot, Scheib, and Haffen, 1977; Tanabe et al., 1979; Woods and Erton, 1978). Early endocrinology experiments by Berthold in 1848 demonstrated that early castration of male chickens resulted in adult male chickens that were not masculinized (Nelson, 2000). However, evidence also suggests that adrenal glands are also steroidogenic in the embryonic chick. Tanabe *et al* (1979) measured steroids in adrenal tissue only, and discovered adrenals were capable of secreting T and E_2 as well as corticosterone (Tanabe et al., 1979). It is suggested that post-hatch, adrenal glands become more specialized, producing mainly corticosteroids rather than sex steroids. Additionally, work has shown that the post-hatch chick brain contains steroids (Migues, Johnston, and Rose, 2002), suggesting that the brain may also be steroidogenic.

In Japanese quail, one study examined serum concentrations of testosterone, 5α-DHT, E_2 and PROG in the second half of embryonic life (E9-17 of incubation) and during the first 5 weeks after hatching. Embryos were blood sampled from a large blood vessel in the egg membranes, while hatchlings were blood sampled via decaptitation. A sex difference was detected in plasma E_2 over most of the age range studied (Schumacher, Sulon, and Balthazart, 1988). T peaked on E15 in females and was significantly higher than in males. In males, T did not peak until 1 week post-hatch, and was significantly higher than in females (Schumacher et al., 1988). Another study examining sex steroids during quail development also found sex differences in androgens and E_2 in blood from the chorioallantoic vein. Males had higher androgens than females at all ages sampled except for the day of hatch, where females had higher androgen levels (Ottinger et al., 2001). In contrast, plasma E_2 levels were higher in females on all days sampled. Studies have also examined circulating sex steroids in each sex specifically. In females, plasma E_2 in the chrorioallantoic vein remained relatively constant in the embryo, followed by a sharp decrease post-hatch in blood collected via decapitation (trunk blood) (Abdelnabi, Bakst, Woods, and Ottinger, 2000). In males, plasma androgens were elevated on E8, declined, and rose again at E13 and remained elevated until P3. Levels then subsequently remained low until after P18 (Ottinger and Bakst, 1981).

It has been suggested that the source of circulating steroids in quail are the gonads (Ottinger et al., 2001), but there is also evidence that the adrenals are also steroidogenic in quail (Ottinger et al., 2001). Males had higher androgen levels in gonads and adrenals than females. Male adrenal glands had higher levels than the testes (Ottinger et al.,

2001). In contrast, females had higher E_2 content in adrenals and gonads compared to males (Ottinger et al., 2001). These results are consistent with the results obtained in the chicken.

Other studies examining sex steroid levels during development in non-passerine birds are in Shao ducks, white storks, and greylag geese. In Shao ducks, blood samples were collected from ducklings at P1, P15 and P35, and then subsequently at 20 day intervals from the wing vein and analysed for T and E_2. Plasma T in males increased throughout the experimental period, while plasma E_2 in females increased to a peak at P135, and then declined afterwards (Yang, Medan, Watanabe, and Taya, 2005). However, no sex differences in circulating sex steroids were assessed. In another study of white storks (*Ciconia ciconia L.*), plasma T levels were measured on P5 and P15. T was higher on P15 than P5, but data were not analysed for sex differences (Sasvari, Hegyi, and Peczely, 1999). In greylag geese (*Anser anser*), excreted metabolites of gonadal steroids were measured. Samples were collected from P10 to P80. Androgen metabolites were significantly higher during the first 20 days of life than during the last 60 days before fledging, while estrogen metabolites during the first 20 days of life were not different than the last 60 days before fledging (Frigerio, Moestl, and Kotrschal, 2001). Data were not analysed for sex differences.

In summary, sex differences in steroids pre- and post-hatch have been characterized in non-passerine birds. Additionally, data suggest that the adrenals may be steroidogenic in development, and may contribute to steroids for sexual differentiation of brain and behaviour during the embryonic and post-hatch stages.

Passerine Birds

Sex steroid levels during development in altricial birds have mainly been characterized in captive zebra finches (*Taeniopygia guttata*). In songbirds, there is a critical period during nestling development, usually during the first week of life (Adkins-Regan, Mansukhani, Seiwert, and Thompson, 1994). During this critical period, song nuclei are sensitive to sex steroids, and exposure to E_2 leads to song nuclei development in males (Gurney and Konishi, 1980). One early study examined levels of AE, T, E_2 and 5α-DHT in jugular plasma of nestling birds, focusing specifically on the critical period in zebra finches (Hutchison, Wingfield, and Hutchison, 1984). Androgens in both sexes were high on hatch date (P0), but had declined by P10. In contrast, plasma E_2 was basal in male hatchlings, but increased substantially to a peak on P4. This peak resulted in a highly significant sex difference in plasma E_2, as female E_2 levels did not change considerably during development. Although no evidence was presented in this study, the authors suggested gonads or adrenals as possible sources for these sex steroids. However, since then, jugular blood has been shown to be enriched with neurally formed steroids (Schlinger and Arnold, 1993).

Further work expanded on this study to include pre-hatch, the second week of life and beyond fledging. Adkins-Regan and colleagues (1990) examined circulating E_2, T or 5α-DHT in developing zebra finches in blood from either trunk blood source, which is a mixture of venous (jugular) and arterial (carotid) blood, or blood from the brachial vein. There was no sex difference detected in circulating steroids during the first week of life (Adkins-Regan, Abdelnabi, Mobarak, and Ottinger, 1990). However, in the second week of life a sex difference in E_2 was detected on P12, with males having higher levels.

Another study in zebra finches examined circulating plasma steroids in pooled trunk and cardiac puncture blood during the nestling phase. Again, plasma AE, T, E_2 estrone (E_1), and 5α-DHT levels were examined, as well as aromatase activity. Blood data were averaged over a range of ages (P1-3, P4-6, etc.). No sex difference was detected in any of the plasma steroids (Schlinger and Arnold, 1992). Moreover, there was no sex difference in aromatase levels in the brain (Schlinger and Arnold, 1992).

Sex steroids have also been examined in free-living passerines. T levels were examined in nestling European starlings (*Sturnus vulgaris*) trunk blood. T increased steadily post-hatch to P8, then decreased towards the end of the nestling period (Williams, Dawson, Nicholls, and Goldsmith, 1987). Moreover, there gonads were small in the nestlings, suggesting that circulating T may be of non-gonadal origin.

Another study examining sex steroids during the nestling phase of wild birds was in Great tits (*Parsus major*). T and E_2 levels were examined in jugular plasma during the nestling phase. High levels of both steroids were present in newly hatched birds of both sexes, declining to basal levels at P3 and P2 for T and E_2, respectively (Silverin and Sharp, 1996). A sex difference was only detected in plasma T on P0 and P1 (where P0 is hatch day), with males having significantly higher plasma levels than females. Electron microscopy examination of male gonads only characterized spermatogonia at all ages examined. Exogenous gonadotropin releasing hormone (GnRH) treatment did not result in T release from the gonads. This further indirectly supports the idea that gonads are not the source of circulating T during development in songbirds.

Plasma E_2 levels were also measured in castrated and intact song (*Melospiza melodia*) and swamp sparrows (*Melospiza georgiana*) from hatch to P200 (Marler,

Peters, Ball, Dufty Jr., and Wingfield, 1988). Levels in castrated and intact birds were similar, suggesting that the source of E_2 in these birds was non-testicular. A similar study in swamp sparrows only measured plasma T and E_2 from 26 days to 1 year of age, the sensitive period for song acquisition and development. T was elevated between 30 and 80 days of age, as well as 260 to 360 days of age, while plasma E_2 was elevated from 18 to 170 days of age, corresponding with low T levels (Marler, Peters, and Wingfield, 1987).

Androgen levels were measured in wild developing African black coucals (*Centropus grillii*), a species with sex-role reversal, as a possible mechanism for behavioural masculinization and subsequent aggression in females. Throughout the nestling phase, plasma androgen levels were higher in males than females (Goymann, Kempenaers, and Wingfield, 2005). Another study examined pied flycatchers (*Ficedula hypoleuca*) at P7. Levels of T were measured in the plasma, but the data were not analysed for a sex difference in T levels (Goodship and Buchanan, 2006).

Alternative Sources for Sex Steroids During Songbird Development

Taken together, these studies suggest that the gonads are not the source of circulating T during songbird nestling development. Moreover, manipulative studies in songbirds have suggested that gonadal steroids may not play a role in sexual differentiation of the songbird brain (Adkins-Regan and Ascenzi, 1990; Arnold, 1975; Wade and Arnold, 1996). The alternative sources may include: the adrenal glands, the liver, and the brain.

Support for steroid synthesis in the adrenals was documented in work done in adult non-breeding song sparrows (Soma and Wingfield, 2001). Detectable levels of the sex steroid precursor, dehydroepiandrosterone (DHEA) were found in plasma (~1 ng/mL), even when the gonads were regressed. DHEA was also detectable in the adrenals (~12 ng/g) and testes (~14 ng/g). DHEA could be released into the plasma and converted to T and E_2 in tissues such as the brain, which possess the appropriate enzymes for these conversions (Soma, Alday, Hau, and Schlinger, 2004; Tam and Schlinger, 2006; Vanson, Arnold, and Schlinger, 1996).

Alternatively, the liver may also be a source of DHEA. Experiments in young rats has demonstrated that the liver expresses P450c17, the enzyme that converts pregnenolone (PREG) to DHEA (Katagiri, Tatsuta, Imaoka, Funae, Honma, Matsuo, Yokoi, Ishimura, Ishibashi, and Kagawa, 1998; Vianello, Waterman, Dalla Valle, and Colombo, 1997). Circulating PREG in the bloodstream may be taken up by the liver and converted to DHEA, which is subsequently released into the bloodstream.

Additionally, the brain is a potential source of steroids. Brain tissue may synthesize steroids *de novo* from cholesterol (Mellon and Griffin, 2002). Brain slices from P25 male zebra finches synthesized E_2 *in vitro* with PROG added to the cell culture media (Holloway and Clayton, 2001). In addition, the developing avian brain possesses the appropriate enzymes to synthesize steroids (London, Monks, Wade, and Schlinger, 2006).

These alternative sources may provide an explanation for the inconsistency in detecting sex differences in circulating sex steroids during development in previous studies. However, few studies mentioned above have directly measured steroids in

tissue, and no previous studies have directly measured steroids in songbird brain or peripheral tissue.

OBJECTIVES

The general objective of this thesis was to employ a comprehensive approach to examine possible sources of steroids (specifically DHEA and E_2) during the nestling phase in songbirds. Using a nestbox breeding colony of free-living European starlings, I explored the following specific objectives:

(1) To determine developmental changes in circulating steroids in songbird nestlings.

(2) To determine sex differences in circulating sex steroids during the nestling phase.

(3) To determine the source of circulating sex steroids during development by:

    a. Comparing hormones in brachial and jugular plasma and

    b. Measuring steroid levels in gonads, adrenals, liver and brain

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Chapter 2: Brain and Plasma Steroid Levels in a Developing Free-Living Songbird[1]

INTRODUCTION

Sexual differentiation of the brain has traditionally been thought to be driven by gonadal hormones, particularly testosterone (T). In rodents, gonadal steroids have an organizational effect on the nervous system during a critical period, directing neural and behavioural development in either the male or female direction (Arnold, 2004; Phoenix et al., 1959). T is thought to be released by the testes and then converted to 17β-estradiol (E_2) in the brain (Figure 2.1). E_2 is important in organizing the male brain and adult male reproductive behaviour.

However, sexual differentiation of the songbird brain may be an exception to this paradigm, because in songbirds, the gonads may not be the sole source of steroids affecting brain development. The nestling phase contains a critical period during which the telencephalic song nuclei are masculinized in males but not in females. In this critical period, the song nuclei are sensitive to sex steroids, particularly E_2 (Gurney and Konishi, 1980). However, removing testes in developing zebra finch males fails to prevent masculinization of the brain (Adkins-Regan and Ascenzi, 1990; Arnold, 1975). Moreover, inducing testicular tissue in embryonic zebra finch females fails to masculinize the brain (Wade and Arnold, 1996). Taken together, these data suggest that gonadal steroids are not the sole determinants for masculinizing song nuclei in males.

Previous work has examined sex steroid levels during development. One study examined androstenedione (AE), T, dihydrotestosterone (DHT) and E_2 in juvenile zebra finches during development and found males had significantly higher E_2 levels than

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[1] A version of this chapter will be submitted for publication and the reference will be as follows: Chin, E.H., K.L. Schmidt, L.D. Sheldon, A.H. Shah, O.P. Love and K.K. Soma, 2006. Brain and plasma steroid levels in a free-living songbird.

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females on post-hatch day 3 (P3, where P0 is hatch day) (Hutchison et al., 1984).

Another study examining AE, T, DHT and E_2 in developing juvenile zebra finches found males had higher E_2 levels than females on P12 (Adkins-Regan et al., 1990). In contrast, another study looking at AE, T, DHT, E_2 and estrone (E_1) in juvenile zebra finches did not find a sex difference in E_2 during development (Schlinger and Arnold, 1992). However, these studies sampled blood from different veins. Hutchison *et al* (1984) collected blood from the jugular vein, whereas Adkins-Regan *et al* (1990) collected blood from the brachial vein, and Schlinger and Arnold (1992) collected blood from cardiac puncture. Additionally, there are no sex differences in estrogen receptor (ER) mRNA, aromatase mRNA (Perlman and Arnold, 2003), androgen receptor (AR) mRNA (Perlman, Ramachandran, and Arnold, 2003), or aromatase activity (Schlinger and Arnold, 1992) during the nestling phase.

Taken together, these data suggest (a) that there may be alternative mechanisms to supply the brain with sex steroids or (b) that sex steroids may play a smaller role in sexual differentiation. One alternative source of steroids is the adrenal gland, which synthesizes sex steroid precursors such as dehydroepiandrosterone (DHEA) (Schlinger, Lane, Grisham, and Thompson, 1999; Soma, Wissman, and Wingfield, 2002). These precursors are subsequently converted locally to E_2 at target tissues, which contain the appropriate enzymes for metabolism, such as a the brain (Tam and Schlinger, 2006; Vanson et al., 1996) (Figure 2.1). Another possible source of sex steroids is the liver, which expresses Cyp17 (17α-Hydroxylase/17,20-lyase), an enzyme required for DHEA synthesis, during development in rats (Katagiri et al., 1998). A third alternative source of sex steroids is the brain where locally formed estrogens influence neural development

(Adkins-Regan et al., 1990; Holloway and Clayton, 2001; London et al., 2006; Schlinger and Arnold, 1992; Schlinger, Soma, and London, 2001). Alternatively, sex steroids may not be solely responsible for sexual differentiation of the brain. It has been hypothesized that expression of sex chromosome genes in the brain drive sexual differentiation in songbirds (Arnold, 2004).

While several studies have examined hormone profiles during development in captive zebra finches, few studies have focused on free-living passerines. European starlings (*Sturnus vulgaris*) are an excellent model for addressing this issue. Predominantly males sing in this species; however, females also sing (Cabe, 1993). Song nuclei are also larger in male starlings (Bernard, Casto, and Ball, 1993). E_2 treatment to female starling chicks partially masculinizes song nuclei (Casto and Ball, 1996). However, Williams *et al* (1987) did not find sex differences in plasma testosterone (T) in European starlings during development.

In the present study, I examined hormone profiles during development in starling nestlings in plasma and tissue. The goals in this study were to: (1) measure DHEA and E_2 in plasma from brachial and jugular veins, and (2) measure steroid concentrations in gonads, adrenals, liver and brain. The jugular plasma is enriched with neurally derived steroids (Schlinger and Arnold, 1993). If the jugular plasma has higher levels of sex steroids, then the brain may be the source of sex steroids during development. If steroid levels in the brachial plasma are higher than the jugular plasma, then peripheral tissues such as the gonads, adrenals or liver may be the source of sex steroids during development. Male starling chicks are predicted to have higher steroid levels than female

starling chicks, as males develop larger song nuclei and sing more frequently than females.

MATERIALS AND METHODS

Field Site and Subjects

This research was carried out between April and July 2005 and 2006 at the Davistead Dairy Farm in Langley, British Columbia, Canada, under a University of British Columbia Animal Care permit (A04-277), following guidelines of the Canadian Council on Animal Care. The site consists of approximately 250 nest boxes mounted on farm buildings and on posts in large fields that are used yearly by breeding starlings. All nest boxes were checked daily to determine clutch initiation and clutch completion dates as well as the laying sequence of eggs and rates of chick growth. Starlings at our field site generally lay 4-6 eggs per clutch, incubate for 10-11 days and fledge chicks 20-24 days following hatching (O.Love, unpublished data). Nestling hatch order and subsequent age were tracked using non-toxic food colouring and chick-specific feather clipping until 10 days of age, when chicks were subsequently banded with metal bands (permit #10765).

Experiment 1: Steroid Levels in Plasma

This experiment was conducted in 2005. We collected blood samples from developing birds from either the jugular or the brachial vein. Day of hatch was considered P0. Juvenile birds were sampled on post-hatching day 0 (P0), P1, P2, P4, P6, P8, P10, P16 and P20 (fledging age). On the day of hatch, nests were assigned two ages at which blood samples were collected. These two ages were at least a week apart. No

bird was blood sampled more than two times. For each blood sample collected from a particular bird, the same vein was sampled at each age. No more than 1% of total blood volume was collected per sample. Brachial blood was accessed by puncture of the brachial vein in the wing with a 26G needle and then collected with a heparinized capillary tube. Jugular blood was collected with heparinized 0.5 mL fixed needle syringes. All samples were collected between the hours of 12:00 and 16:00 and within 5 min (2.75 ± 0.07 min) of initial disturbance to avoid effects of stress. After blood collection, we obtained body measurements (mass, and length of exposed culmen, metatarsus, and wing) from the bird and returned it to its nestbox. Wing cord measurements were taken from P8 and older birds when primary feathers began to appear. Blood was centrifuged at 10,000 rpm for 10 min. Plasma was collected with a Hamilton syringe and stored at -20°C until analysis.

Experiment 2: Steroid Levels in Brain Tissue

This experiment was carried out in 2006. We collected tissue from juvenile starlings at P0, P6 and P8 based on previous data from (Silverin and Sharp, 1996), Casto (unpublished data) and (Williams et al., 1987) on plasma steroid levels in wild developing songbirds. All subjects were collected between the hours of 12:00 and 16:00 and sacrificed within 3 min of initial disturbance. Before sacrifice, a blood sample was collected from the jugular vein using a heparinized 0.5 mL syringe. Birds were then rapidly decapitated, and the brain and body were chilled at -20°C for 5 min prior to dissection. Brain and peripheral tissues were collected and frozen in liquid nitrogen. For the brain, the telencephalon was divided into rostral telencephalon (rTEL), caudal

telencephalon (cTEL), and dorsal telencephalon (dTEL). The telencephalon was not subdivided in P0 chicks.

The dTEL contained the song nuclei HVC and NCM, as well as the hippocampus. In P1 chickens, the dTEL contains IMHV, where there are high levels of DHEA (Migues et al., 2002). To dissect out the dTEL, cuts were made on the telencephalon in the following places: halfway between rostral and caudal edges, halfway between dorsal and ventral edges, and halfway between the midline and lateral edges.

The rTEL contained the song nuclei lMAN and Area X. To dissect out the rTEL, after the dTEL was collected, tissue from the rostral edge to halfway between the rostral and caudal edges was considered rTEL and collected. The cTEL contained the song nucleus RA. After the rTEL was collected, tissue from halfway between the rostral and caudal edges to the caudal edge of the brain was considered cTEL and collected.

The body was kept at 4°C while the brain was dissected. The liver, heart, gonads and adrenals were dissected out of the body. All dissections were finished within 30 min (26.87 ± 0.48 min) of initial disturbance.

Steroid Extraction from Plasma

Extraction methods using organic solvents did not yield a serial dilution that was parallel to the radioimmunoassay standard curve. Therefore, steroids were extracted using solid phase extraction (SPE) with C18 columns (6 mL capacity, 500 mg, non-endcapped; United Chemical Technologies UCTCUC18156). Either 66 μL or 132 μL of plasma were prepared for extraction by bringing samples to 10 mL with deionized water (diH$_2$O). Using a vacuum manifold, each column was primed with 3 mL HPLC-grade ethanol, followed by 10 mL diH$_2$O. We then added the 10 mL sample volume. Next, the

columns were washed with 10 mL diH$_2$O to remove substances that may interfere with the radioimmunoassay. Steroids were eluted with 5 mL 90% HPLC-grade methanol into 7 mL borosilicate glass scintillation vials. Each sample was evaporated to dryness under N$_2$ at 37°C. Depending on initial plasma volume, samples were resuspended in either 440 μL or 880 μL of phosphate buffered saline with gelatin, PBSG (5% ethanol, final concentration). 100% ethanol (absolute grade) was added first to the bottom of the vial and briefly vortexed to bring steroids into solution. PBSG was then added to bring samples up to final volume. These volumes were determined from preliminary studies and designed to place samples in the linear portion of the standard curve. Samples were then shaken at 500 rpm for 1 hr and stored overnight at 4°C. The next morning, samples were again shaken at 500 rpm for 2 min prior to radioimmunoassay. To quantify recovery of DHEA and 17β-E$_2$, separate samples from a pool of juvenile starling plasma were spiked with 50 pg DHEA and 0.5 pg 17β-E$_2$, then compared to unspiked plasma. Recovery of DHEA was 104.89 ± 6.09% (n = 4 pairs), and recovery of 17β- E$_2$ was 107.45 ± 13.90% (n = 4 pairs). Therefore, plasma concentrations of DHEA and E$_2$ were not corrected for recovery.

Steroid Extraction from Tissue

Steroids were also extracted from tissue using solid phase extraction (SPE). The procedure for brain tissue was the same as that for plasma, except tissue required more preparation prior to loading the samples on the columns. Tissue was weighed and transferred to 13 x 100 mm borosilicate glass test tubes. Ice-cold diH$_2$O was added (3x times the weight of the tissue). Tissue was homogenized on ice using a Teflon pestle. Immediately, HPLC-grade methanol was added (4x the total volume of tissue and water).

Samples were sonicated for 15 min (Fisher Scientific FS-20), shaken for 60 min (IKA Vibrax VXR basic), and stored overnight at 4°C. The next day, samples were shaken for 90 min, and then centrifuged at 3000g for 10 min at 2°C. The supernatant was transferred to 20 mL scintillation vials. In cases where the supernatant volume was greater than 1 mL, only 1 mL of supernatant was transferred to the scintillation vial. The supernatant was brought up to 10 mL with diH$_2$O. This 10 mL volume was loaded onto primed and equilibrated C18 columns. Columns were washed with 10 mL of diH$_2$O. Steroids were eluted with 5 mL 90% HPLC-grade methanol into 7 mL borosilicate scintillation vials. Each sample was evaporated to dryness under N$_2$ at 37°C. Samples were resuspended in 440 µL PBSG (5% ethanol, final concentration). To quantify recovery of DHEA and 17β-E$_2$, separate samples from a pool of juvenile starling tissue were spiked with 50 pg DHEA and 1 pg 17β-E$_2$, then compared to unspiked tissue. From brain, recovery of DHEA was 143.02 ± 10.42% (n = 5 pairs), and recovery of 17β- E$_2$ was 61.59 ± 10.68% (n = 5 pairs). From adrenal tissue, recovery of DHEA was 50.61 ± 6.14% (n = 5 pairs), and from gonadal tissue, 60.37 ± 22.22% (n = 5 pairs). All tissue concentrations were corrected for recovery and were expressed as pg steroid per g tissue wet weight.

Radioimmunoassays

DHEA was measured using a double antibody RIA which uses ^{125}I-DHEA as the tracer (Diagnostic Systems Laboratories, DSL-8900). The DHEA assay was modified as per Granger et al (1999), and has been previously used with songbirds (Goodson, Evans, and Soma, 2005). Briefly, using PBSG, supplied DHEA standards were diluted 10x, the primary antibody was diluted 4x, and the tracer was diluted 4x. The DHEA antibody has a low cross-reactivity with DHEA-S (0.02%), 16β-OH DHEA (0.041%), androstenedione

(0.46%), testosterone (0.028%), and E_2 (< 0.004%; E. Chin, unpublished data). Assay sensitivity was 2 pg/tube.

17β-E_2 was measured using a double antibody RIA which uses ^{125}I-17β-E_2 as the tracer (Diagnostic Systems Laboratories, 3rd generation E_2 RIA, DSL-39100). The 17β-E_2 assay was modified as per Shirtcliff $et\ al$ (2000). Briefly, using PBSG, supplied E_2 standards were diluted 20x, the primary antibody was diluted 4x, and the tracer was diluted 3x. The 17β-E_2 antibody has a low cross-reactivity with estrone (6.9%), estriol (< 0.001%), 17α-E_2 (0.33%; E. Chin, unpublished data), and DHEA-S (< 0.001%). Assay sensitivity was 0.113 pg/tube.

Genotyping

Once plasma was collected from the blood samples, a portion of the remaining red blood cells was transferred to a piece of filter paper, which was then stored at -20°C. Starling chicks were genotyped using a polymerase chain reaction (PCR) amplification process based on techniques used by (Griffiths, S. Daan, and Dijkstra, 1996) and (Chin, Love, Clark, and Williams, 2005). 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. Adult birds of known sex (n = 5 of each sex) were used as positive controls. Negative controls contained no DNA. PCR products were run on a 3% agarose gel with ethidium bromide at 70V for 70 min. DNA was visualized with a UV transilluminator.

Statistics

The plasma data were subjected to a two-way analysis of variance (ANOVA) followed by planned *post hoc* t-tests. If necessary, data were normalized by logarithm (base 10) transformation prior to running the ANOVA. Jugular plasma data from 2005 and 2006 were pooled for analysis following t-tests which determined that there was no statistically significant year difference. Plasma samples with non-detectable steroid levels were assigned the detection limit for the assay. To analyze for sex differences rather than regional differences, the tissue data were subjected to paired t-tests, or Mann-Whitney U-tests if the data had unequal variance. All tests were performed using Sigma Stat 3.0 with nestling age and sex as factors. Statistical significance was set at $p = 0.05$. Sample sizes for all groups are presented in Table 2.1. Results are presented as mean ± standard error of the mean.

RESULTS

Subject Body Mass and Hematocrit

Changes in body mass with age are depicted in Figure 2.2. Male starlings hatched at 6.38 ± 0.26g and fledged at 73.61 ± 1.09g, and female starlings hatched at 6.02 ± 0.30g and fledged at 70.81 ± 1.28g. For both sexes, mass gain in juvenile starlings increased in a linear fashion from hatch to P10, after which mass subsequently plateaued to fledging. Hematocrit increased from 22.8 ± 2.0% at hatch to 47.5 ± 1.1% at fledging for both sexes (Table 2.2).

Validations

Initial validations of a serial dilution of starling plasma using organic solvents such as dichloromethane failed to yield a serial dilution that was parallel to the standard curve (Figure 2.3a). However, serial dilutions of starling plasma and brain tissue pools extracted using SPE yielded parallelism to both the DHEA and 17β-E_2 standard curves (Figures 2.3, 2.4). All water blanks run during extractions were non-detectable or close to the lowest point on the standard curve for both DHEA (2.63 ± 0.24 pg; n = 15) and 17β-E_2 (< 0.113 pg; n = 10). There was no effect of 100% ethanol (absolute grade) concentration for resuspension (5% or 10% final concentration) on the RIAs, thus 5% ethanol (final concentration) was chosen to resuspend samples (Figure 2.5, 2.6).

Plasma Steroid Levels

Plasma DHEA. All brachial DHEA samples were detectable (Figure 2.7a). For brachial DHEA, there was a main effect of age (F = 3.711; p < 0.001), but not sex (F = 1.344; p = 0.248). There was no interaction between age and sex (F = 1.793, p = 0.084). For juvenile males, brachial DHEA levels ranged from ~1000 pg/mL during the first few days of life and peaked at ~2400 pg/mL at P4. Levels declined back to ~1000 pg/mL by P20. For juvenile females, DHEA levels were ~1000 pg/mL at hatch and increased to ~1500 pg/mL at P2. Levels then declined back to 1200 pg/mL by P20. Males had 1.5 times more DHEA than females at P4, and this sex difference was highly significant (t = 3.289; p = 0.001).

All jugular DHEA samples were detectable (Figure 2.7b). For jugular DHEA, there was a main effect of age (F = 2.339, p = 0.021) and sex (F = 5.333; p = 0.022). There was also a significant interaction between sex and age (F = 2.268; p = 0.025). For

juvenile males, jugular DHEA levels were low at hatch, 600 pg/mL, compared to brachial levels. For juvenile females, jugular DHEA was considerably higher at hatch, ~1100 pg/mL, and peaked at ~1500 pg/mL by P10. In contrast to brachial DHEA data, females had higher levels of DHEA in jugular plasma than males on P0 (t = 3.105; p = 0.002) and P10 (t = 2.329; p = 0.021).

To compare differences between veins, the average brachial DHEA levels at each age were subtracted from each individual jugular DHEA levels for that age within each sex (Figure 2.7c). There was a highly significant main effect of age (F = 8.501; p < 0.001), and sex (F = 21.648; p < 0.001). There was also a highly significant interaction between age and sex (F = 5.714; p < 0.001). *Post hoc* t-tests revealed a sex difference at P0 (t = 3.155; p = 0.002), P4 (t = 4.866; p < 0.001), and P10 (t = 4.638; p < 0.001) with females having higher difference in DHEA between the jugular than brachial vein than males.

Plasma E_2. In comparison, plasma E_2 levels were 100 fold lower than DHEA levels in both sexes. E_2 was not detectable in all brachial samples (Table 2.3a). For brachial E_2, there was a main effect of age (F = 2.245; p = 0.028) but not sex (F = 1.344; p = 0.249) (Figure 2.8a). There was no significant interaction between age and sex (F = 0.484; p = 0.865). For juvenile males, brachial E_2 levels were mainly non-detectable at hatch and rose to 15 pg/mL by P1. Levels dropped back to non-detectable levels at P2, but rose again to 24 pg/mL by P4. Levels subsequently declined to non-detectable by P20. For juvenile females, brachial E_2 levels were also mainly non-detectable at hatch and followed a similar pattern to male levels throughout development. Levels in females

reached 18 pg/mL at P4 before declining to non-detectability by P20. There was no sex difference in brachial E_2 levels at any age.

For jugular E_2, not all samples were detectable (Table 2.3a). There was a main effect of age ($F = 9.475$; $p < 0.001$), but not sex ($F = 0.0441$; $p = 0.834$) (Figure 8b). There was no significant interaction between sex and age ($F = 0.576$; $p = 0.797$). For juvenile males, levels were non-detectable at hatch, and increased to 17 pg/mL at P4. Levels declined to 10 pg/mL by P8 and remained low to P20. For juvenile females, the pattern was similar. Levels were non-detectable at hatch, peaked at 20 pg/mL at P4, and then declined to 10 pg by P8 and remained low to P20. There was no sex difference in jugular E_2 levels at any age.

E_2 levels were also compared between the jugular and brachial veins (Figure 2.8c). There was also a highly significant main effect of age ($F = 12.567$; $p < 0.001$) and a significant main effect of sex ($F = 8.041$; $p = 0.005$). The interaction between sex and age was also highly significant ($F = 3.553$; $p < 0.001$). *Post hoc* t-tests revealed females having higher difference in E_2 between the jugular than brachial vein than males at P4 (t $= 3.577$; $p < 0.001$), P6 (t $= 3.414$; $p < 0.001$) and P8 (t $= 3.770$; $p < 0.001$).

Plasma E_2/DHEA Ratio. To determine how much DHEA was being metabolized to E_2, the ratio of E_2 to DHEA levels for each subject was compared, potentially indicating the amount of DHEA converted to E_2 (Figure 2.9). For brachial steroid levels, there was no effect of age ($F = 1.835$; $p = 0.077$) or sex ($F = 0.0518$; $p = 0.820$). There was also no significant interaction between age and sex ($F = 1.149$; $p = 0.336$). For jugular steroid levels, there was an effect of age ($F = 3.663$; $p < 0.001$), but

not sex (F = 3.211; p = 0.075). There was no significant interaction between age and sex (F = 1.430; p = 0.187).

Brain Steroid Levels

P0. At P0, DHEA levels in the brain were detectable in all regions of the brain (Figure 2.10a). There was no sex difference in DHEA in the telencephalon (t = -0.084; p = 0.934) or the optic lobe (t = 0.362; p = 0.723). There was a sex difference detected in the diencephalon, with males having higher DHEA levels than females (t = -2.161; p = 0.048).

P6. At P6, DHEA was also detectable in all regions of the brain (Figure 2.10b). There was no sex difference in DHEA levels in any of the brain regions measured: rostral telencephalon (t = -0.043; p = 0.966), caudal telencephalon (t = 1.557; p = 0.142), dorsal telencephalon (Mann Whitney, t = 76.00; p = 0.442), diencephalon (t = 0.594; p = 0.562) and optic lobes (Mann Whitney, t = 70.00; p = 0.878).

P8. At P8, DHEA was also detectable in all brain regions (Figure 2.10c). There was no sex difference in DHEA levels in any of the brain regions measured: rostral telencephalon (t = -1.157; p = 0.267), caudal telencephalon (t = -0.639; p = 0.533), dorsal telencephalon (t = -0.750; p = 0.466), diencephalon (t = -0.268; p = 0.793), and optic lobes (Mann Whitney, t = 58.00; p = 0.328).

Brain E_2 Levels. Surprisingly, E_2 was not detectable in any brain region at any age. The detection limit for all ages is presented in Table 2.3. However, positive controls were run to ensure that the extraction technique was efficient. E_2 was extracted and measured in adult zebra finch ovary, and the adult male zebra finch brain. E_2 was

detectable in all samples of adult zebra finch ovary and adult male zebra finch brain, as well as spiked brain tissue samples.

Peripheral Tissue DHEA Levels

P0. At P0, DHEA was detectable in all tissues collected (Figure 2.11a). There was no sex differences detected in any of the tissues measured: gonads (Mann Whitney; t = 45.00; p = 1.00), adrenals (t = -1.079; p = 0.299), and liver (Mann Whitney; t = 63.00; p = 0.645). The heart (negative control) had lower levels of DHEA than the jugular plasma. E_2 was not measured because plasma levels were low at this age.

P6. At P6, DHEA was detectable in all tissues collected (Figure 2.11b). There was no sex difference in DHEA levels in tissues collected: gonads (Mann Whitney; t = 53.00; p = 0.130), adrenals (t = -0.193; p = 0.849) or liver (Mann Whitney; t = 62.00; p = 0.574). The heart (negative control) had lower levels of DHEA than the jugular plasma. E_2 was not measured because plasma levels were low.

P8. At P8, DHEA was detectable in all tissues (Figure 2.11c). There was a sex difference detected in the gonads, with the testes containing higher DHEA levels than the ovaries (Mann Whitney; t = 46.00; p = 0.021). There was no sex difference detected in the adrenals (t = -0.643; p = 0.531) or the liver (t = 0.506; p = 0.621). The heart (negative control) had lower levels of DHEA than the jugular plasma. E_2 was not measured because plasma levels were low.

DISCUSSION

This study investigated the changes in steroid levels during development in a free-living songbird. Assessing plasma DHEA and E_2 levels revealed higher DHEA levels

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than E_2 levels. Males had higher DHEA levels than females at P4 in the brachial vein, while in contrast, females had higher DHEA levels than males at P0 and P10 in the jugular vein. No sex difference was detected in either vein at any age for E_2. In the brain, DHEA levels were low, and a sex difference was only detected at P0 in the diencephalon. E_2 was not detectable in the brain at any age or either sex. In the periphery, DHEA was detectable in all tissues. This is the first study to directly measure steroids in plasma, brain and peripheral tissues from songbirds.

Plasma DHEA and E_2

In the brachial plasma, males exhibited higher levels of DHEA than females on P4. The timing of elevated levels of DHEA in males corresponds to timing of elevated E_2 levels in previous work done with zebra finches (Hutchison et al., 1984). In addition, this peak in DHEA occurs during the first week of life, which has been suggested to be the critical period for sexual differentiation of the song nuclei, as previously determined in zebra finches (Adkins-Regan et al., 1994). Moreover, this suggests there is a peripheral source of DHEA.

In jugular plasma, the opposite sex difference was observed at P0 and P10, with the females having higher levels than the males. This may suggest that males may take up and metabolize DHEA in the brain more than females. This may be linked to differential enzyme activity in the songbird brain. Previous work has demonstrated that there is a sex difference in 3β-Hydroxysteroid dehydrogenase (3β-HSD) in the adult songbird brain, which converts DHEA to AE (Soma et al., 2004) (Figure 2.1). This enzyme is also present in the developing songbird brain (Schlinger and London, 2006).

Should there also be a sex difference during development, this may result in differential metabolism of DHEA, accounting for the higher levels of DHEA in the females.

To further elucidate the idea that the brain takes up DHEA synthesized in the periphery, the average brachial DHEA for each sex and age group was subtracted from each jugular DHEA value for that sex and age group. The difference between the two veins was representative of the steroid levels the brain removed from the plasma. At P0 and P10, females had higher difference in DHEA between the jugular than brachial vein than males, suggesting that the female brain at these ages were not taking up DHEA synthesized in the periphery. Moreover, at P4, the males had less DHEA in the jugular plasma compared to the brachial, suggesting the P4 male brain was taking up DHEA. Moreover, females had higher jugular than brachial levels of E_2 from P2 to P6, suggesting the male brain was also taking up E_2 during this period. These ages fall within the critical period during which the brain is sensitive to steroids (Adkins-Regan et al., 1994; Gurney and Konishi, 1980).

The low ratio of E_2 to DHEA suggests that the DHEA produced and circulated in the blood either (1) was not being converted to E_2 or (2) that the E_2 was being further converted downstream to other estrogens. This is surprising as E_2 has been suggested to be the primary sex steroid responsible for brain sexual differentiation in birds based on elevating levels of E_2 during the critical period (Adkins-Regan et al., 1994; Casto and Ball, 1996; Gurney and Konishi, 1980). This may suggest that further metabolism of E_2 is required during sexual differentiation.

Circulating high levels of DHEA during development may be a physiological strategy employed to avoid the negative impacts of high circulating levels of T. DHEA

can only affect tissues which possess the enzymes to convert DHEA further downstream to T and E_2. In contrast, high circulating T has been connected to decreased immune function and increased metabolic rate (Owen-Ashley, Hasselquist, and Wingfield, 2004; Wikelski, Lynn, Breuner, Wingfield, and Kenagy, 1999), both of which are important physiological factors for juveniles. By circulating DHEA, only target tissues will be affected by T or E_2, and such important physiological factors will not be impacted during development. It has been shown that DHEA does not highly impact immune function (Owen-Ashley et al., 2004).

Brain DHEA and E_2 Levels

DHEA was detectable in all brain regions at all ages. However, there was no sex difference detected in any region at any age. Moreover, DHEA levels were lower in the brain than in plasma. There was also only one sex difference in DHEA levels detected: at P0, in the diencephalon. The results of the current study suggest that the brain may be metabolizing DHEA. DHEA may be converted to E_2. However, E_2 measurements in the brain in this study do not indicate that this is the case. Previous work has demonstrated that the brain contains enzymes to metabolize DHEA (London et al., 2006; Soma et al., 2004). Another possibility may be that DHEA is converted into another estrogen. Previous work looking at human pregnancy has suggested that the fetal adrenal produces the sulfated ester, DHEA-S. This DHEA-S is hydroxylated at the 16α position by the fetal liver, and then aromatized to estriol (E_3) by the placenta. It is conceivable that perhaps a similar process is occurring in the juvenile songbird brain, and that the sex difference may lie in E_3, not DHEA or E_2 (Diczfalusy, 1984).

Surprisingly, E_2 was not detectable in any brain regions at any age, despite detectable levels in circulating plasma. This does not support the hypothesis that E_2 is primarily responsible for brain sexual differentiation, and further supports the findings of low E_2 in circulation (Casto, 2001). There may be several explanations for the low levels of E_2 in the brain. Firstly, E_2 may be further converted to other estrogens, such as estrone (E_1) or estriol (E_3). E_1 is detectable in plasma in developing songbirds, and levels are higher than circulating E_2 (Schlinger and Arnold, 1992). E_1 may be the active estrogen in the brain after conversion from E_2, which may explain why levels in the plasma are also quite low. Secondly, the method of dissections of the brain may have diluted out high concentrations of E_2. Small punches of brain tissue may reveal more localized concentrations of E_2. Thirdly, 17β-E_2 may not be the active estrogen in the brain. A recent study has suggested that the isomer, 17α-E_2 is a neuroactive estrogen (Toran-Allerand, Tinnikov, Singh, and Nethrapalli, 2005). This is the first study to measure steroid levels in songbird brain tissue, and further work is needed to determine if E_2 levels in the brain are more localized.

Peripheral DHEA

DHEA was detectable in all tissues at all ages. Particularly, DHEA levels were high in the gonads, 50 to 200 times higher than the plasma, depending on age. This suggests that the gonads are the primary source of peripheral DHEA. Previous studies have suggested that the gonads are not sources of sex steroids required for brain sexual differentiation (Wade and Arnold, 1996), and that gonadal tissue did not actively synthesize steroids during the nestling phase (Schlinger and Arnold, 1992; Silverin and Sharp, 1996). However, these studies only considered T and E_2 levels, and did not

consider the capacity of the gonads to synthesize DHEA from cholesterol. The data in the current study suggest that these tissues have Cyp17 activity during the nestling phase, the enzyme required to convert PREG to DHEA (Figure 2.1). These data support the findings from previous studies which have found that the gonads express Cyp17 mRNA (Freking, Nazairians, and Schlinger, 2000). It is possible that while the gonads do not possess all the enzymes for synthesizing T and E_2 during the critical period, they may still be capable of synthesizing and releasing DHEA into circulation, which can be subsequently converted at target tissues. Moreover, the induced testes in juvenile female zebra finches may have secreted DHEA, but the brain did not have the appropriate enzymes to convert the DHEA further downstream to affect brain anatomy.

DHEA was also detectable in the adrenals, suggesting that the adrenals may contribute to peripheral levels of DHEA. It has been previously demonstrated that the avian adrenal is capable of synthesizing DHEA (Soma and Wingfield, 2001). The adrenals contribute to peripheral levels of DHEA in wintering song sparrows, when the gonads are regressed and are not synthesizing T. Juveniles are physiologically similar to wintering songbirds, as their gonads are regressed. It is possible that the adrenals may be a secondary source of DHEA. In the event that gonads are regressed or removed all together, the adrenals may take over as the primary source of DHEA. This may explain why castration had no effect on song behaviour in male zebra finches (Arnold, 1975). Taken together, the data support the idea that the gonads and adrenals secrete DHEA, which may be converted to T and E_2 at target tissues.

DHEA was also detectable in the liver, but not at levels higher than the plasma. Previous studies in developing rodents have suggested that the liver is capable of

synthesizing DHEA as there is Cyp17 activity in the liver during development (Katagiri et al., 1998; Tagawa et al., 2005). While it is possible that the liver is a source of DHEA during development, further studies should examine possible Cyp17 activity in the avian liver. The liver is a large organ in developing songbirds, and may express large amounts of Cyp17. The whole liver was not collected here, and DHEA levels in the liver were low. Further studies should examine Cyp17 activity as well as DHEA content of the entire liver.

The results of this study both support new hypotheses as well as challenge older ones. Firstly, the high levels of DHEA in the gonads and adrenals support the hypothesis that the adrenals secrete DHEA into the bloodstream to be subsequently converted at target tissues. Secondly, high levels of DHEA detected in the gonads suggest that the gonads do play a role in sex steroid synthesis during development and are not completely inactive. Thirdly, the non-detectable levels of E_2 in the brain suggest that E_2 may not be the active estrogen in the brain during development. However, these data do not rule out the hypotheses that the brain is capable of synthesizing steroids, or that there may be a direct genetic effect on sexual differentiation of the brain. Further studies should focus on other potential active estrogens in the brain, more localized measurements of E_2 in the brain, as well as enzyme activity, such as Cyp17 and 3β-HSD, in tissues analyzed in this study.

Tables

Table 2.1: Sample sizes for all groups for (a) 2005 and (b) 2006 experiments.

(a)

Age	Males		Females	
	Brachial	Jugular	Brachial	Jugular
P0	6	7	4	7
P1	12	10	5	11
P2	8	9	4	7
P4	9	8	11	8
P6	8	7	13	7
P8	8	6	4	8
P10	7	7	8	8
P16	10	13	9	7
P20	9	10	9	7

(b)

Age	Males	Females
P0	8	8
P6	8	8
P8	8	8

Table 2.2: Changes in hematocrit over the nestling phase in juvenile European starlings with sexes combined. Values are presented as means ± SEM.

Age	Hematocrit (%)	Sample Size
P0	22.7 ± 2.0	5
P1	23.7 ± 1.5	4
P2	21.9 ± 2.0	14
P4	24.4 ± 1.6	8
P6	34.1 ± 1.5	17
P8	36.6 ± 2.1	16
P10	36.8 ± 1.8	20
P16	40.8 ± 0.8	18
P20	47.4 ± 1.0	22

Table 2.3: Number of detectable samples for E_2 in (a) plasma and (b) brain tissue in juvenile European starlings. Values are presented as means ± SEM and number of detectable samples in brackets. Detection limit for plasma was 9 pg/mL. Detection limit for brain tissue is presented in the table.

(a)

Age	Males		Females	
	Brachial	Jugular	Brachial	Jugular
P0	0/6	1/7	1/4	1/7
P1	6/12	4/10	4/5	3/11
P2	6/8	6/9	3/3	5/7
P4	9/9	7/8	11/11	8/8
P6	8/8	7/7	7/13	5/7
P8	6/8	2/6	3/4	3/8
P10	3/7	3/7	4/8	7/8
P16	6/10	7/13	3/9	5/7
P20	5/9	4/10	8/9	3/7

(b)

Age	Sex	TEL	rTEL	cTEL	dTEL	DIEN	OL
P0	Female	32.2 ± 1.6 (0/8)				65.7 ± 6.9 (0/8)	36.4 ± 5.6 (0/8)
	Male	31.7 ± 1.2 (0/8)				77.1 ± 10.6 (0/8)	39.4 ± 5.4 (0/8)
P6	Female		23.3 ± 2.4 (0/8)	26.3 ± 3.5 (1/8)	23.4 ± 2.7 (0/8)	30.1 ± 3.8 (1/8)	21.6 ± 2.1 (0/8)
	Male		28.8 ± 5.0 (0/8)	22.4 ± 3.2 (0/8)	26.8 ± 5.8 (0/8)	32.1 ± 4.7 (0/8)	46.1 ± 25.5 (0/8)
P8	Female		17.9 ± 0.5 (0/8)	18.9 ± 0.4 (0/8)	17.0 ± 0.4 (0/8)	22.4 ± 4.7 (2/8)	20.8 ± 1.4 (0/8)
	Male		21.5 ± 2.9 (0/8)	21.2 ± 1.4 (0/8)	19.0 ± 0.9 (0/8)	19.5 ± 1.1 (0/8)	19.1 ± 0.6 (0/8)

Figure 2.1: Pathways of steroid synthesis. Adapted from Mellon and Griffin, 2002.
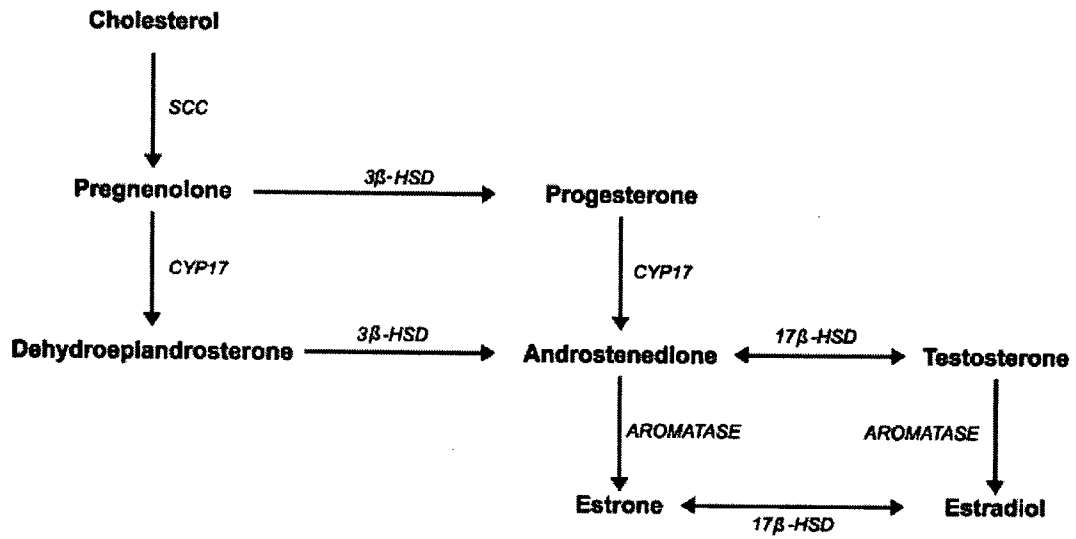
Figure 2.2: Changes in body mass during development in European starlings.
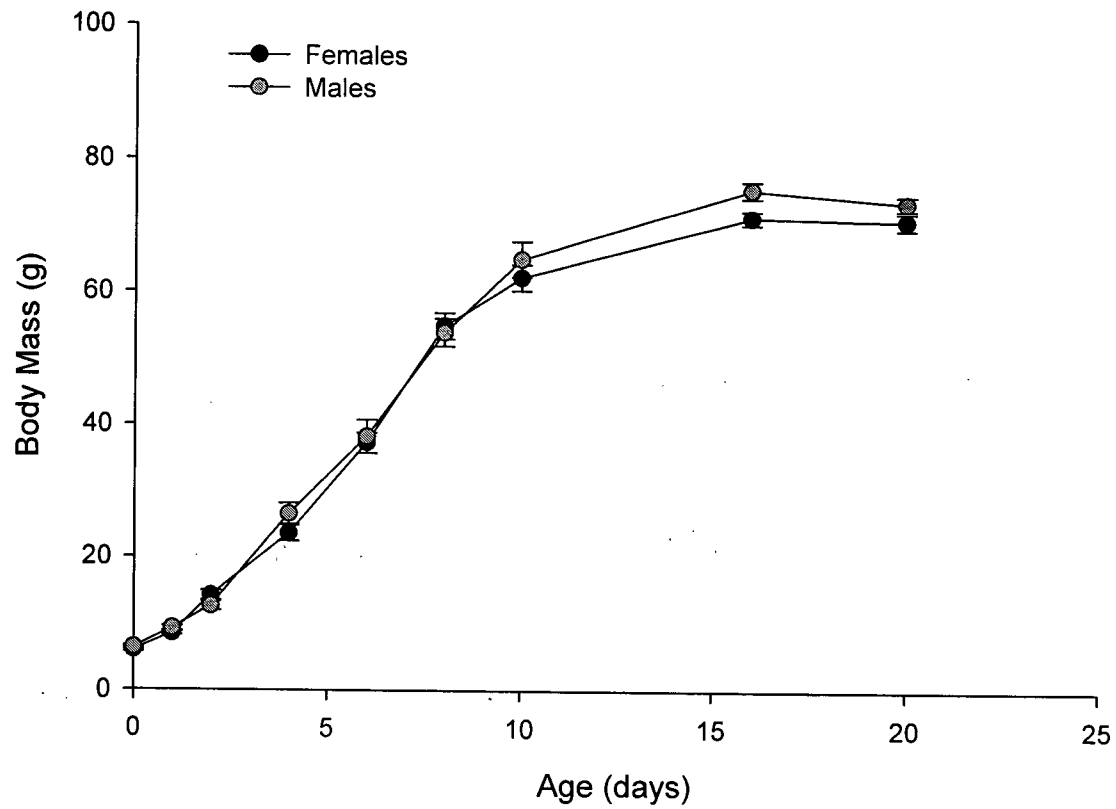
Figure 2.3: Steroid extraction from juvenile starling plasma using (a) dichloromethane to extract DHEA, (b) solid phase extraction (SPE) to extract DHEA and (c) SPE to extract 17β-E$_2$. Circles represent the standard curve for each assay, and triangles are the serial dilution of juvenile starling plasma. Results are plotted on a log scale x-axis.

(a)
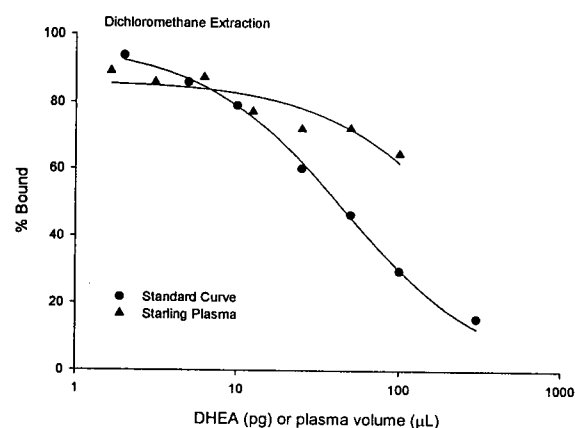
(b)
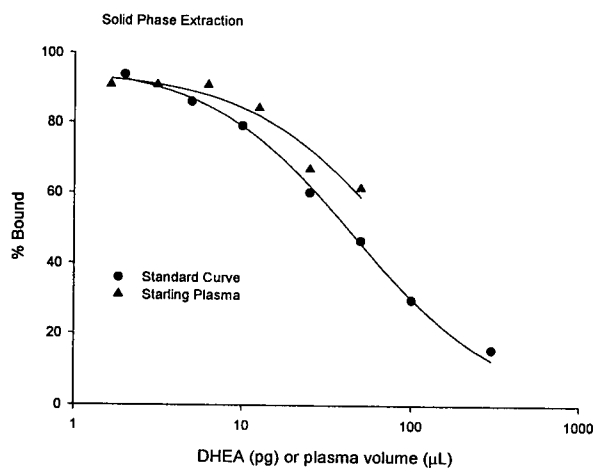
(c)
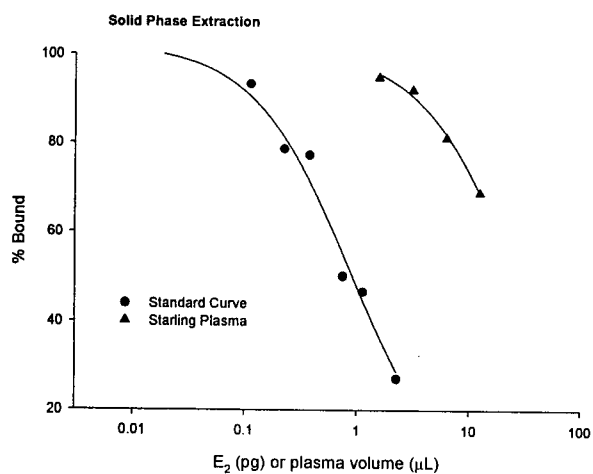
Figure 2.4: Steroid extraction from juvenile starling brain tissue using SPE. Circles represent the standard curve for each assay, and triangles are the serial dilution of juvenile starling brain tissue.
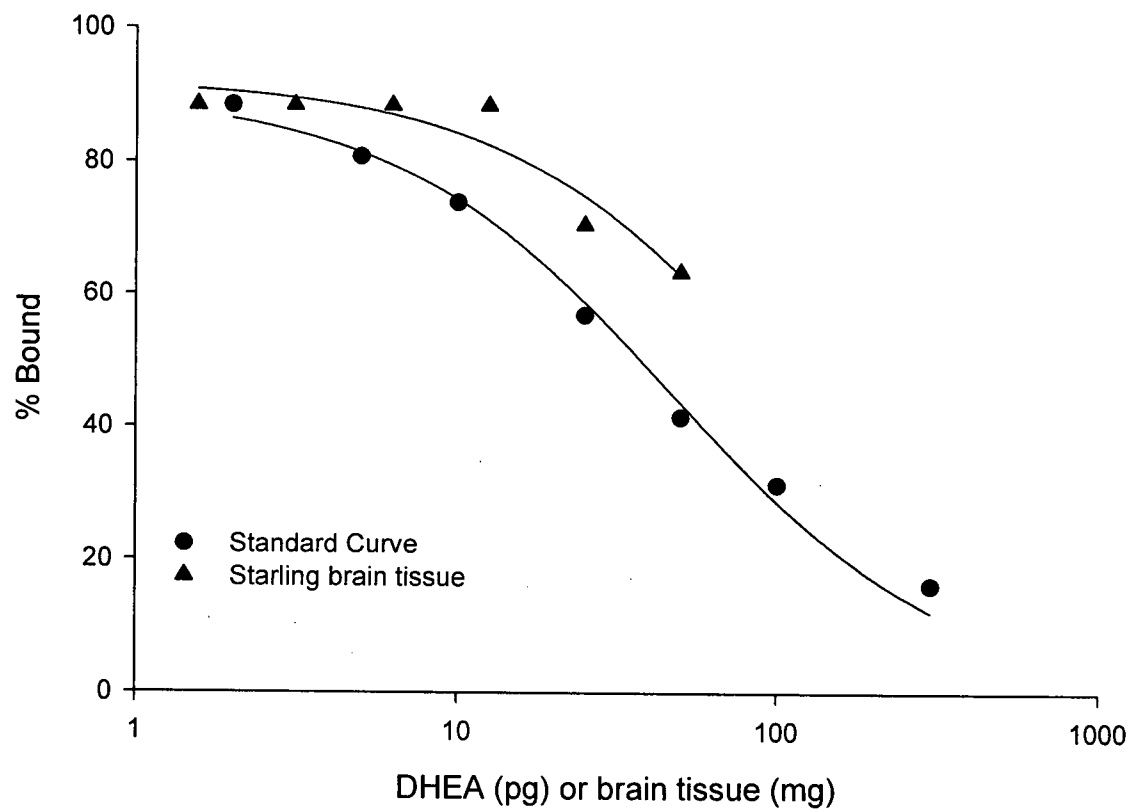
Figure 2.5: Estimating recovery of steroids from avian plasma using SPE with (a) labeled steroids and (b) unlabelled steroids.

(a)
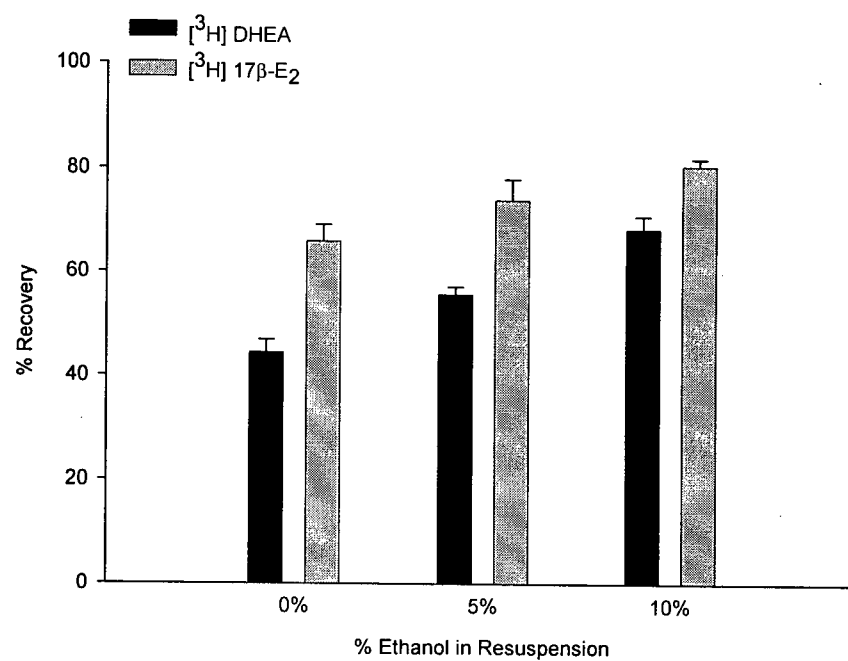
(b)
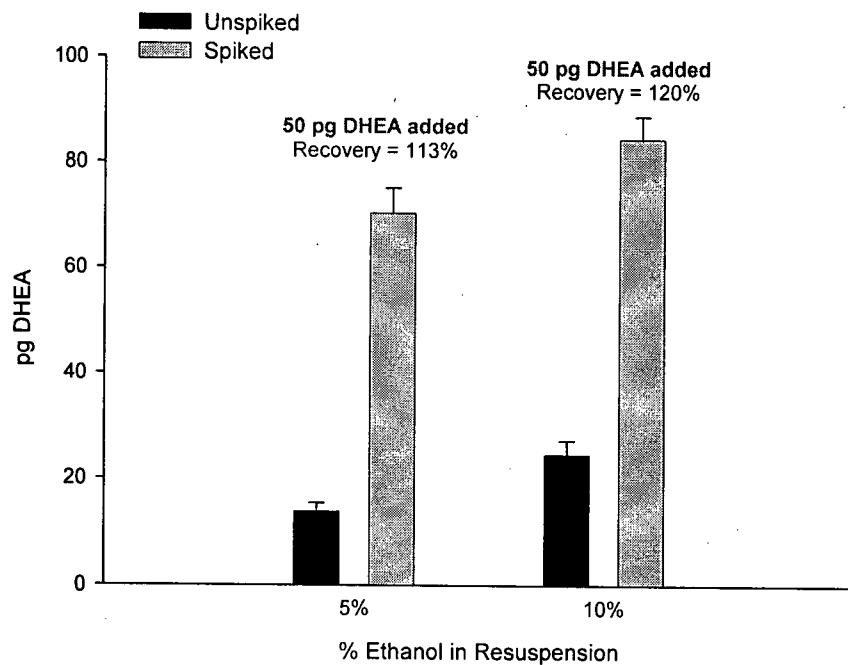
Figure 2.6: Estimating recovery of steroids from avian brain tissue using SPE with (a) labeled steroids and (b) unlabelled steroids. 5% ethanol, final concentration, was used in resuspension.

(a)
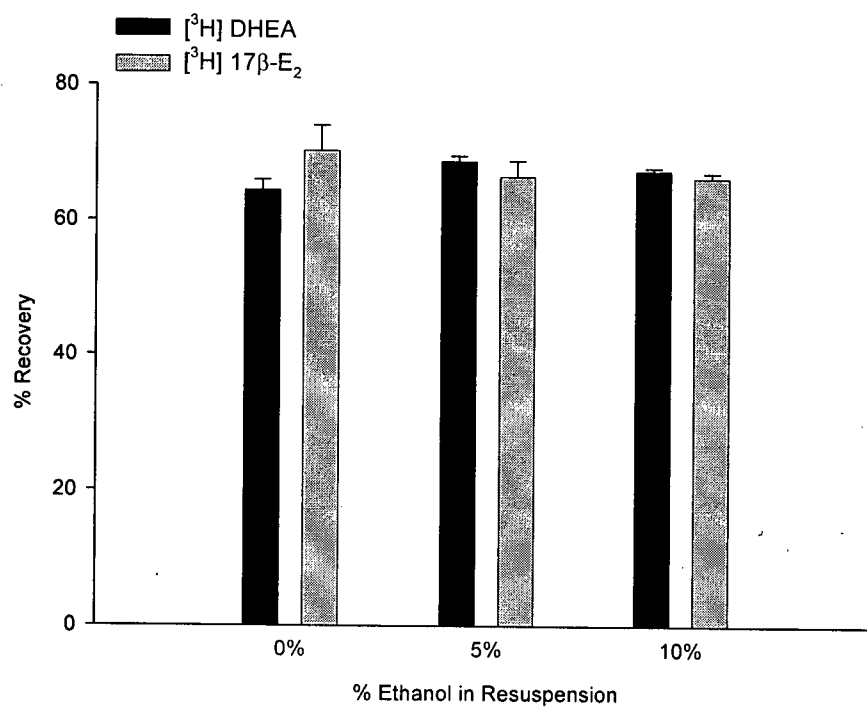
(b)
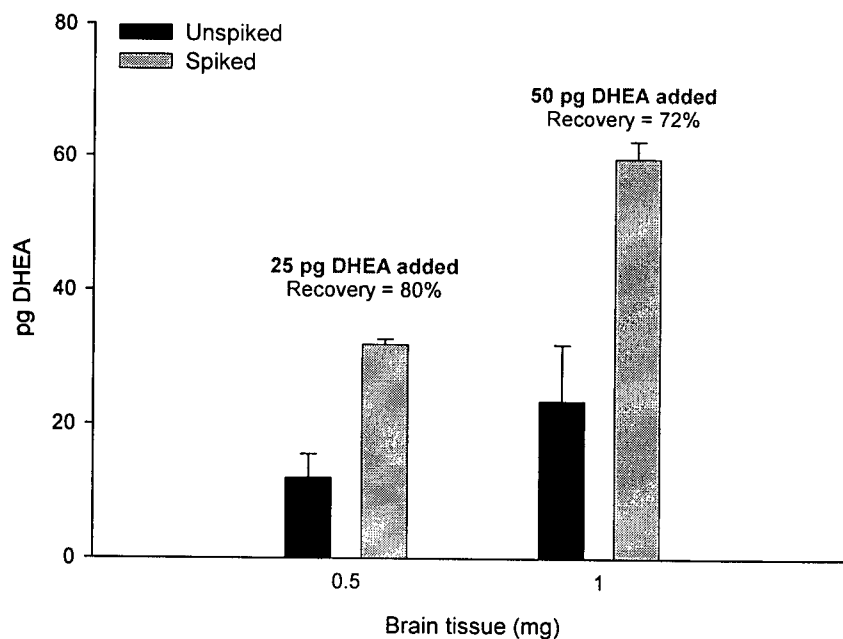
Figure 2.7: DHEA levels in (a) brachial plasma (b) jugular plasma and (c) jugular –
brachial levels in juvenile starlings from hatch (0) to fledging (20). Females are
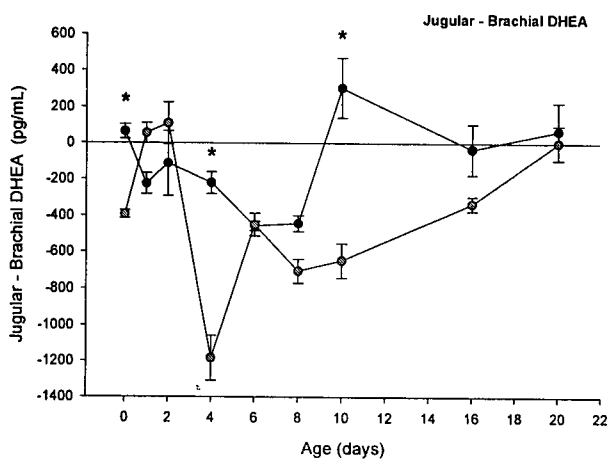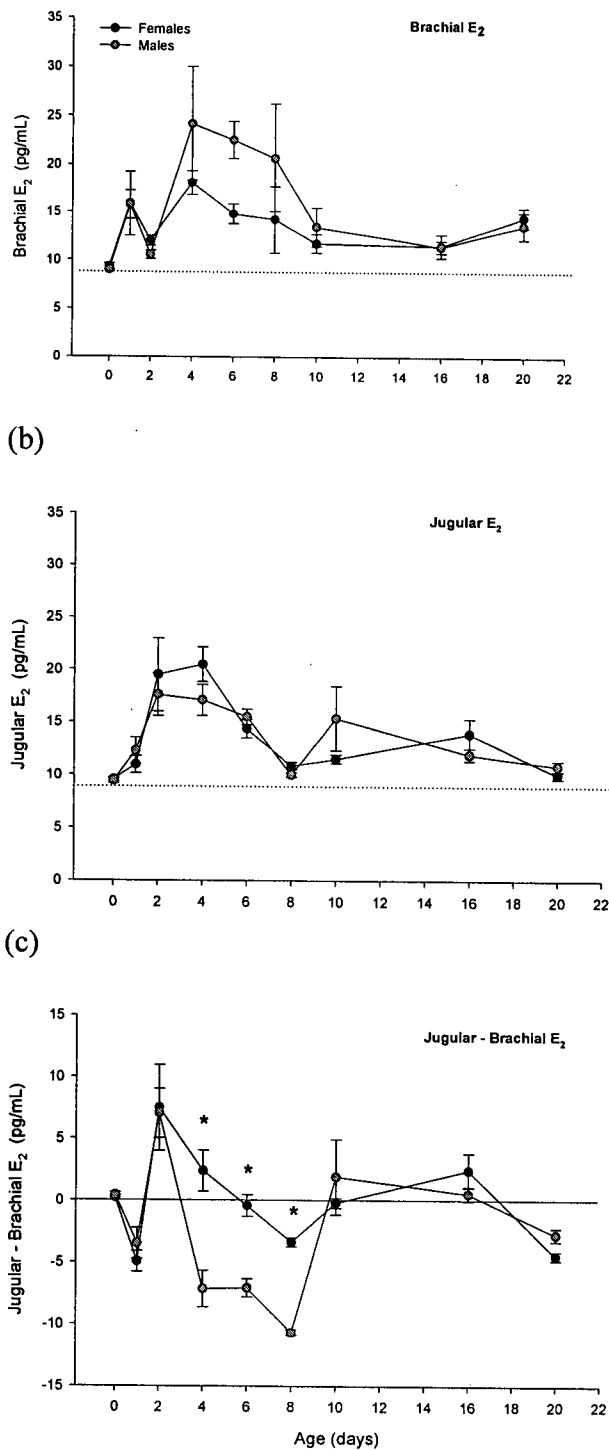represented by black circles, and males are represented by gray circles.
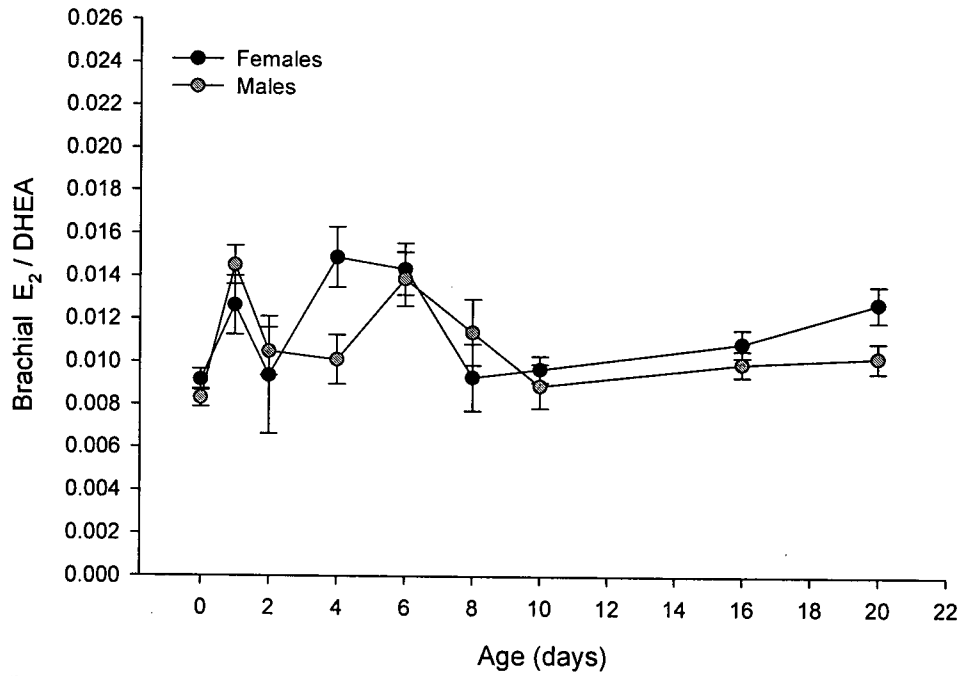
(a)

(b)

(c)
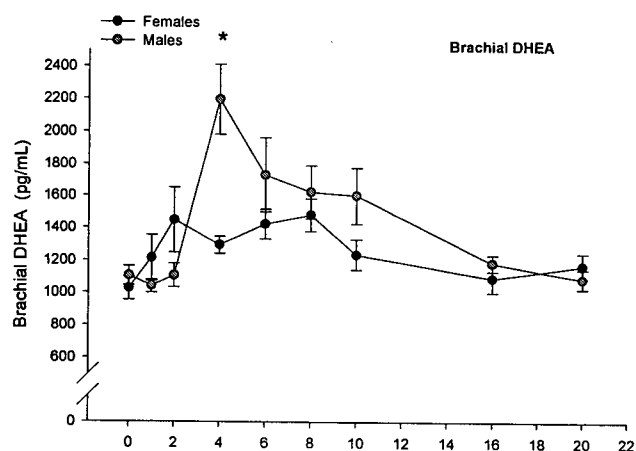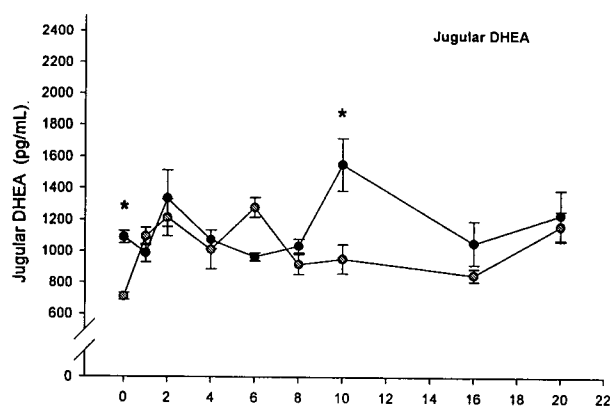
Figure 2.8: 17β-E_2 levels in (a) brachial plasma (b) jugular plasma and (c) jugular – brachial levels in juvenile starlings from hatch (0) to fledging (20). Females are represented by black circles, and males are represented by gray circles.
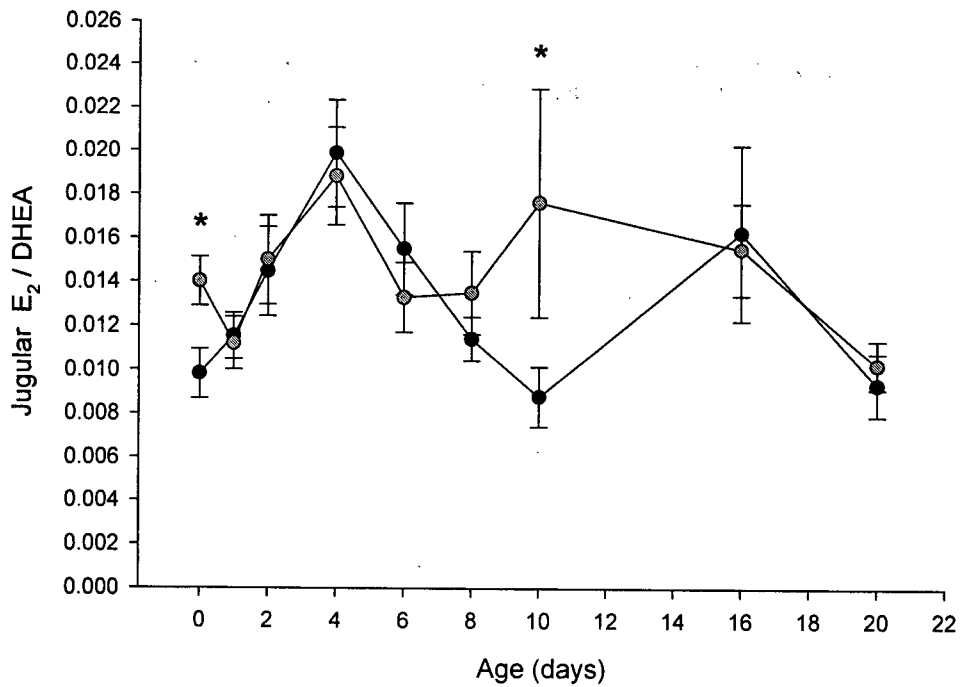
(a)

(b)

(c)

Figure 2.9: E_2/DHEA ratios in (a) brachial plasma and (b) jugular plasma in juvenile starlings at each age sampled.
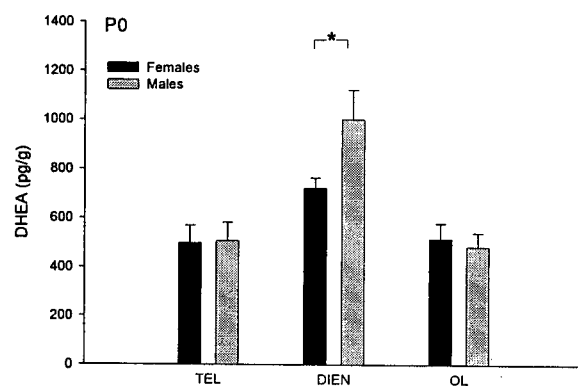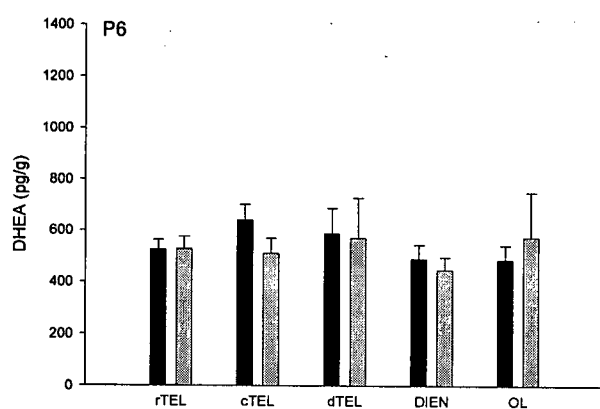
(a)

(b)

Figure 2.10: Brain DHEA levels in (a) P0 chicks (b) P6 chicks and (c) P8 chicks. Samples sizes are n = 8 for both sexes at all ages. Regions measured included telencephalon (TEL) (P0 chicks only), rostral telecephalon (rTEL), caudal telencephalon (cTEL), dorsal telencephalon (dTEL) (P6 and P8 chicks only), diencephalon (DIEN) and optic lobes (OL) (all chicks).
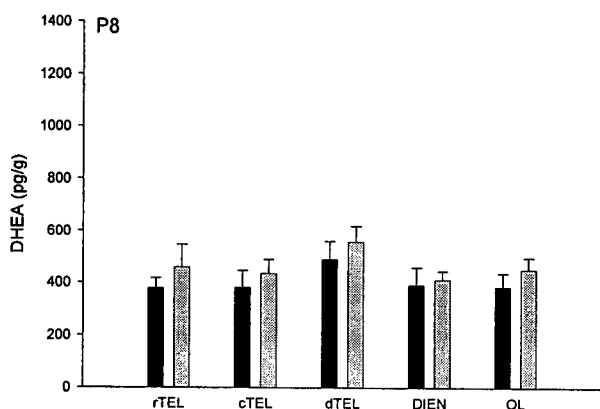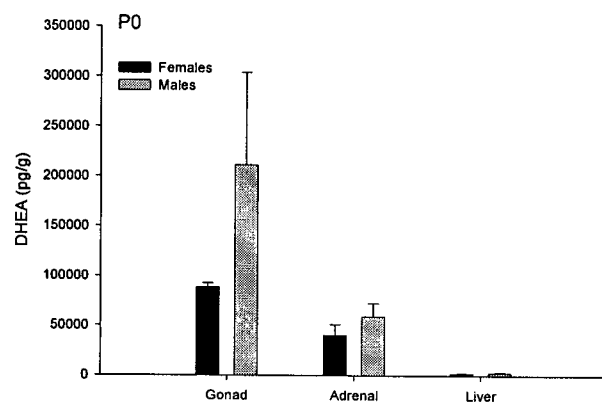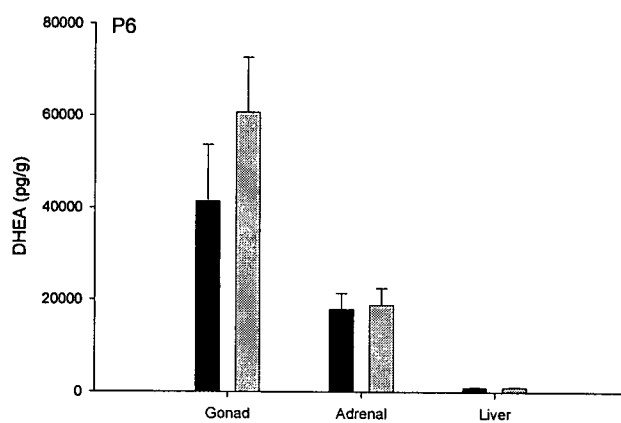
(a)

(b)

(c)

49

Figure 2.11: Periphery DHEA levels in (a) P0 chicks (b) P6 chicks and (c) P8 chicks. Sample sizes are n = 8 for both sexes at all ages.
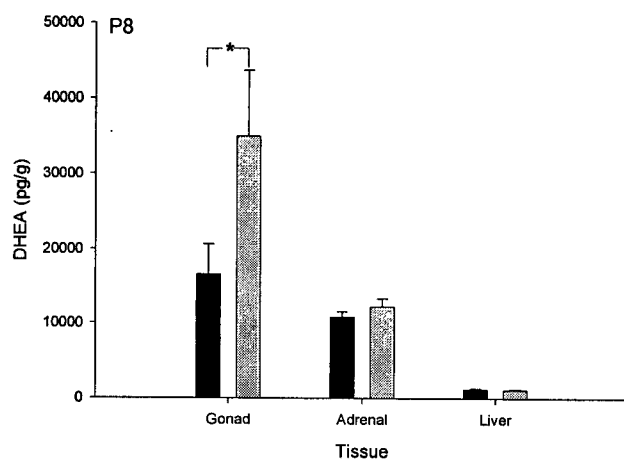
(a)

(b)

(c)

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Chapter 3: General Discussion and Conclusions

The goals of this study were to characterize plasma DHEA and E_2 levels in male and female starling chicks during development, as well as measure steroids in the songbird brain and peripheral tissues in selected ages during development. The findings of this study both support the hypothesis regarding alternative sources of steroids, as well as challenge the idea that the gonads do not play a role in steroid synthesis during development. Previous work has focused on the gonads secreting T, which is converted in the brain to E_2. However, the data from this thesis indicate that DHEA is released by the gonads into the blood, suggesting that the gonads may play a role in steroid synthesis during development.

Previous work in this field has focused primarily on 17β-E_2 as the primary steroid responsible during sexual differentiation of the brain – particularly because administering exogenous E_2 to juvenile female songbirds during the critical period yields a partial masculinization in adulthood (Adkins-Regan et al., 1994; Casto and Ball, 1996; Gurney and Konishi, 1980). However, this study revealed that plasma levels of E_2 are low during development. It is possible that it is not E_2 levels that are important, but the metabolism of E_2 downstream that plays a role in sexual differentiation of the brain.

While this is the first study to examine steroid levels in the songbird brain and peripheral tissue, there are still more studies to pursue. Low levels of DHEA and non-detectable levels of E_2 in the brain were surprising. However, it is possible that levels of DHEA and E_2 are more localized in the brain, and this effect was diluted out by the dissection method employed. Another possibility is that DHEA is being converted to another estrogen, such as 17α-E_2, E_1 or E_3 in the brain. Future studies should focus on

taking more specific areas of the brain, such as HVC, NCM or RA, as well as measuring other estrogens in tissue and plasma that were not previously considered as 'active'. Moreover, only part of the liver was analyzed for DHEA during this experiment. As the avian liver is quite large, future studies should examine the whole liver for DHEA synthesis.

In addition, tissue samples should be collected from more ages during development. This study focused on a limited number of ages, and several key important ages were missed, such as P4 and P10. The ages chosen in this study were made *a priori* based on from previous studies (Casto, 2001; Hutchison et al., 1984; Silverin and Sharp, 1996; Williams et al., 1987). However, the results of this study suggest that a more intensive sampling during development may be useful.

The findings of this study support the hypothesis that there are alternative sources of sex steroids, but challenge the idea that the gonads are inactive during the nestling phase. However, this study does not rule out the hypotheses that the brain is steroidogenic (neurosteroids) or that there may be effects of sex chromosome genes on the brain during sexual differentiation. Future studies should examine these two possibilities and their roles during sexual differentiation of the songbird brain.

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