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Sex differences in DHEA and estradiol during development in a wild songbird: Jugular *versus* brachial plasma

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ABSTRACT

Sexual differentiation of the brain has traditionally been thought to be driven by gonadal hormones, particularly testosterone (T). Recent studies in songbirds and other species have indicated that non-gonadal sex steroids may also be important. For example, dehydroepiandrosterone (DHEA) – a sex steroid precursor that can be synthesized in the adrenal glands and/or brain – can be converted into active sex steroids, such as 17β-estradiol (E_2), within the brain. Here, we examine plasma DHEA and E_2 levels in wild developing European starlings (*Sturnus vulgaris*), from hatch (P0) to fledging (P20). Blood samples were collected from either the brachial vein ($n=143$) or the jugular vein ($n=129$). In songbirds, jugular plasma is enriched with neurally-synthesized steroids and, therefore, jugular plasma is an indirect measure of the neural steroidal milieu. Interestingly, brachial DHEA levels were higher in males than females at P4. In contrast, jugular DHEA levels were higher in females than males at P0 and P10. Brachial E_2 levels were higher in males than females at P6. Surprisingly, jugular E_2 levels were not high and showed no sex differences. Also, we calculated the difference between brachial and jugular steroid levels. At several ages, jugular steroid levels were lower than brachial levels, particularly in males, suggesting greater neural metabolism of circulating DHEA and E_2 in males than females. At a few ages, jugular steroid levels were higher than brachial levels, suggesting neural secretion of DHEA or E_2 into the general circulation. Taken together, these data suggest that DHEA may play a role in brain sexual differentiation in songbirds.

Introduction

Traditionally, sexual differentiation of the nervous system has been thought to be controlled by gonadal sex steroids, particularly testosterone (T) (Arnold, 2004). Support for this hypothesis comes from numerous studies of mammals, primarily laboratory rats, mice, and guinea pigs (McCarthy and Konkle, 2005; Phoenix et al., 1959). Sex differences in the sexually dimorphic nucleus of the preoptic area (Dohler et al., 1984), anteroventral periventricular nucleus (Simerly, 2002), and spinal nucleus of the bulbocavernosus (Morris et al., 2004) are organized in early development by gonadal T. In rats, metabolism of gonadal T to 17β-estradiol (E_2) within the brain is critical for sexual differentiation of the brain during the neonatal period (Simerly, 2002).

In contrast, in songbirds, studies of primarily laboratory zebra finches (*Taeniopygia guttata*) suggest that gonadal T does not regulate sexual differentiation of the telencephalic song system (Wade and Arnold, 1996; Agate et al., 2003). Early E_2 treatment to females partially masculinizes the telencephalic song system (Gurney and Konishi, 1980). However, there are no consistent sex differences in circulating T, brain aromatase, or brain estrogen receptors during development (Schlinger and Arnold, 1992; Perlman and Arnold, 2003). Furthermore, castration of developing male zebra finches fails to prevent masculinization of the song system (Arnold, 1975; Adkins-Regan and Ascenzi, 1990). In addition, inducing functional testicular tissue in developing female zebra finches fails to masculinize the brain (Wade and Arnold, 1996). Lastly, in organotypic slice cultures of brain tissue, sex differences in growth of projections within the song system persist (Holloway and Clayton, 2001). Thus, non-gonadal sex steroids may be important for sexual differentiation of the songbird brain (Schlinger et al., 2001). Alternatively, expression of sex chromosome genes in the brain may regulate sexual differentiation (Agate et al., 2003; Chen et al., 2005a).

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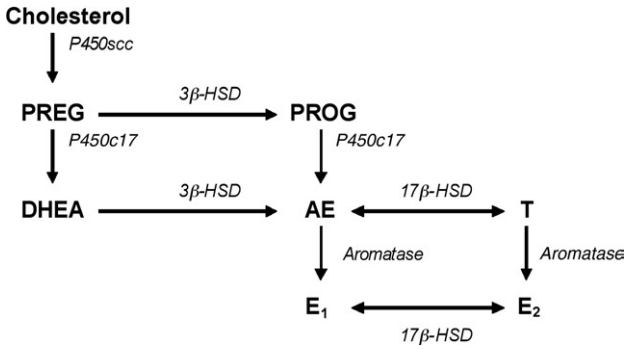


Fig. 1. A simplified diagram of sex steroid synthesis. Steroids: PREG = pregnenolone; PROG = progesterone; DHEA = dehydroepiandrosterone; AE = androstenedione; T = testosterone; E_1 = estrone; E_2 = 17β-estradiol. Enzymes: *P450scc* = Cytochrome P450 side chain cleavage; *P450c17* = Cytochrome P450 17α-hydroxylase/C17,20 lyase; *3β-HSD* = 3β-hydroxysteroid dehydrogenase/isomerase; *17β-HSD* = 17β-hydroxysteroid dehydrogenase; *Aromatase* = Cytochrome P450 aromatase.

Non-gonadal sex steroids could be synthesized in the adrenal glands and/or brain of developing songbirds. There is evidence that the songbird adrenals synthesize dehydroepiandrosterone (DHEA), a sex steroid precursor (Schlinger et al., 1999; Soma and Wingfield, 2001). DHEA can be converted to T and E_2 (Fig. 1) within the brain (Vanson et al., 1996; Soma et al., 2004; Tam and Schlinger, 2007; Pradhan et al., 2008; Schlinger et al., 2008). DHEA treatment affects song behavior and song system anatomy in both adult and juvenile songbirds (Soma et al., 2002; Grisham et al., 2005). Alternatively, the brain may synthesize sex steroids *de novo* from cholesterol to regulate sexual differentiation (Schlinger et al., 2001; London et al., 2006). Lastly, in newly hatched birds, the yolk sac could also be a source of steroids (Williams et al., 2005).

Interestingly, there are no consistent sex differences in circulating E_2 in developing songbirds, although most studies have focused on laboratory zebra finches. In one study of zebra finches, plasma E_2 levels were much higher in males than females on post-hatch day 3 (P3, where P0 is the day of hatch) (Hutchison et al., 1984). A second study in zebra finches found higher plasma E_2 levels in males than females on P12 (Adkins-Regan et al., 1990). A third study in zebra finches did not find any sex differences in plasma E_2 during development (Schlinger and Arnold, 1992). These discrepancies might be explained by the method of blood collection. Hutchison et al. (1984) collected blood from the jugular vein; Adkins-Regan et al. (1990) collected blood from the brachial vein; and Schlinger and Arnold (1992) collected blood via cardiac puncture. In songbirds, jugular blood is enriched with neurally-synthesized steroids, such as E_2 (Schlinger and Arnold, 1993), which may explain the data of Hutchison et al. (1984).

Very few studies have examined sex differences in hormones during development in wild songbirds (Silverin and Sharp, 1996). To do so, European starlings (*Sturnus vulgaris*) are an excellent model species. In this species, both sexes sing, but males sing more than females, and sing more complex and longer songs than females (Cabe, 1993; Pavlova et al., 2005). Song nuclei are larger in adult males than females (Bernard et al., 1993). E_2 treatment to female starling chicks during the first week after hatching (implanted at P3) partially masculinizes song nuclei (~25% increase in HVC volume) (Casto and Ball, 1996). However, Williams et al. (1987) did not find sex differences in plasma T in developing European starlings.

We examined plasma steroid levels during development in wild European starlings. Our goals were to (1) measure DHEA and E_2 in plasma from the brachial and jugular veins, and (2) examine sex differences in DHEA and E_2 levels. In songbirds, jugular plasma is enriched with neurally-synthesized steroids (Schlinger and Arnold, 1993). For DHEA, if the brain synthesizes DHEA during development, then DHEA levels may be higher in jugular plasma than brachial plasma. If the adrenals synthesize DHEA, then DHEA levels may be higher in brachial plasma. For E_2, given the high expression of aromatase in the developing songbird brain (Schlinger and Arnold, 1992), E_2 levels may be higher in jugular plasma, particularly in males.

Materials and methods

Field site and subjects

Blood samples were collected from April to July 2005 at the Davistead Dairy Farm in Langley, British Columbia, Canada, under a University of British Columbia Animal Care permit (A04-277) and following the guidelines of the Canadian Council on Animal Care. The field site contains approximately 250 nest boxes mounted on farm buildings and posts. All nest boxes were checked daily to determine clutch initiation and clutch completion dates. 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 (Chin et al., 2005; Love et al., 2005). Nestlings were individually marked using non-toxic food coloring and feather clipping until 10 days of age, when chicks were banded with numbered aluminum bands (permit #10765).

Field protocol

We collected blood samples from developing birds from either the jugular or the brachial vein. The site of blood collection was randomly assigned. Day of hatch was defined as post-hatching day 0 (P0). Juvenile birds were sampled on P0, P1, P2, P4, P6, P8, P10, P16 and P20. Starling chicks fledge the nest at P20. On P0, nests were randomly assigned two ages for blood collection, and these two ages were at least one week apart. For each subject, blood was collected from the same vein at both ages. No bird was bled more than twice. Each blood sample was <1% of total body mass (e.g., 50 μL blood at P0, 200 μL blood at P4, and 600 μL blood at P10) (sample sizes in Table 1).

For brachial blood samples (n=143 total), we used a sterile 26 gauge needle for venipuncture and collected blood into heparinized microhematocrit tubes. For jugular blood samples (n=129 total), we used sterile heparinized 0.5 mL syringes with fixed needles (28.5 gauge) (Lanctot, 1994; Sheldon et al., in press). Cotton and gentle pressure were used to stop blood flow. Samples were collected between 12:00 and 16:00 to minimize possible diel changes in hormones. Samples were collected within 5 min of initial disturbance (mean±SEM=2.75±0.07 min) to minimize possible effects of stress. Blood was stored at 4 °C until centrifugation (10,000 rpm for 10 min). Then plasma was collected with a Hamilton syringe and stored at −20 °C until analysis.

After blood collection, we obtained body measurements (mass, and lengths of exposed culmen, metatarsus, wing) and returned subjects to their nestboxes. Wing cord measures were taken starting at P8, when primary feathers begin to appear.

Solid phase extraction

Plasma samples appeared lipemic upon visual inspection and traditional steroid extraction methods using organic solvents (diethyl ether, dichloromethane, hexane: ethyl acetate) did not yield serial dilutions that were parallel to the assay standard curve, suggesting that the plasma contained substances that interfered with the hormone assays (Newman et al., 2008).

Therefore, steroids were extracted using solid phase extraction (SPE) with C18 columns (6 mL capacity, 500 mg, non-endcapped; United Chemical Technologies UCTCUC18156) as described previously (Newman et al., 2008). Briefly, plasma samples (66 μL or 132 μL) were brought to a 10 mL volume with deionized water (diH_2O) and added to C18 columns (that were already primed and equilibrated). Samples were washed with diH_2O (5 mL×2) to remove interfering substances. Then steroids were slowly eluted with 5 mL of 90% methanol (HPLC-grade) into 7 mL glass scintillation

Table 1
Sample sizes at each age and vein

Age	Males		Females	
	Brachial plasma (*N* after pooling)	Jugular plasma (*N* after pooling)	Brachial plasma (*N* after pooling)	Jugular plasma (*N* after pooling)
P0	17 (6)	14 (7)	9 (4)	15 (7)
P1	19 (12)	10	8 (5)	11
P2	10 (8)	10	7 (3)	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

Note: If individual samples were pooled, the total number of samples after pooling is in parentheses.

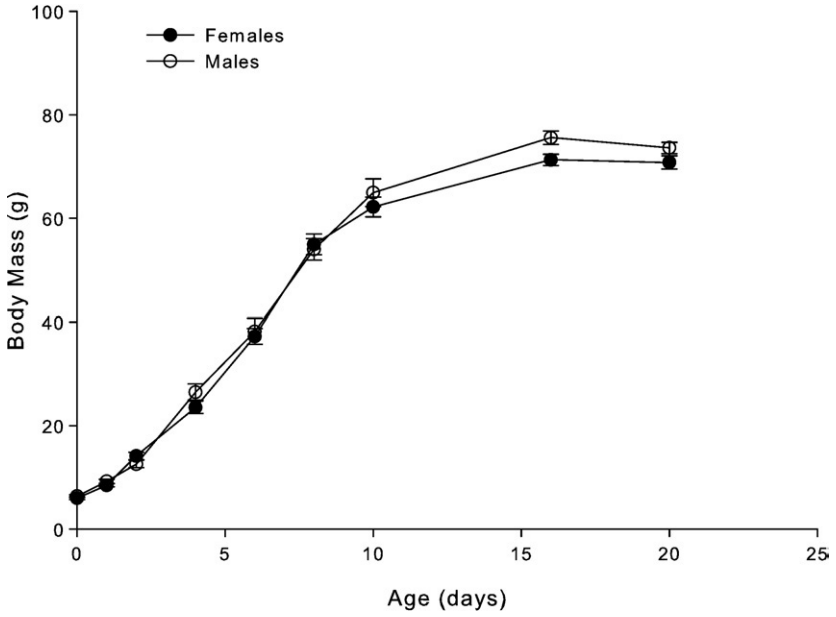


Fig. 2. Changes in body mass during development in wild female and male European starlings.

vials. Samples were taken to dryness under N_2 in a waterbath (~40 °C). Depending on initial plasma volume, samples were resuspended in either 440 μL or 880 μL of phosphate buffered saline with gelatin (PBSG). To aid in resuspending steroids, we used absolute ethanol (5% of resuspension volume), which did not affect the DHEA and E_2 assays used here (Newman et al., 2008). Plasma volumes were based on preliminary studies.

To quantify recovery of DHEA and E_2, separate samples from a pool of juvenile starling plasma were spiked with 50 pg DHEA and 0.5 pg E_2 and then compared to unspiked plasma. Recovery of DHEA was 104.89±6.09% (n=4 pairs), and recovery of E_2 was 107.45±13.90% (n=4 pairs).

Radioimmunoassays

DHEA in samples was measured in duplicate using a double antibody RIA that uses ^{125}I-DHEA as the tracer (Diagnostic Systems Laboratories, DSL-8900). 100 μL of resuspension in duplicate was used (which contained 15 μL of plasma). This DHEA RIA has been used for songbird plasma (Goodson et al., 2005; Soma, 2006; Newman et al., 2008, in press). The DHEA assay was modified to greatly increase sensitivity as described previously (Granger et al., 1999). Briefly, using PBSG, supplied DHEA standards were diluted 10×, the primary antibody was diluted 4×, and the tracer was diluted 4×. The DHEA antibody has low cross-reactivity with DHEA-sulfate (0.02%), 16β-OH DHEA (0.041%), androstenedione (0.46%), testosterone (0.028%), and E_2 (<0.004%; E. Chin, unpublished data). Interassay variation was 11.4% and the lowest point on the standard curve was 2 pg DHEA/tube. All water blanks (n=8 total, 1 per assay) were non-detectable for DHEA (<2 pg).

E_2 in samples was measured in duplicate using a double antibody RIA that uses ^{125}I-E_2 as the tracer (Diagnostic Systems Laboratories, 3rd generation E_2 RIA, DSL-39100). 83.33 μL of resuspension (in duplicate) was used (which contained 12.5 μL of plasma) and brought up to final sample volume (300 μL) with PBSG. This E_2 RIA has been used for songbird plasma (Newman et al., 2008). The E_2 assay was modified to greatly increase sensitivity, as described previously (Shirtcliff et al., 2000). Briefly, using PBSG, supplied E_2 standards were diluted 20×, the primary antibody was diluted 4×, and the tracer was diluted 3×. The E_2 antibody has a low cross-reactivity with estrone (6.9%), estrone-sulfate (<0.01%), estrone-3-glucuronide (<0.01%), estriol (<0.01%), 17α-E_2 (0.33%; E. Chin, unpublished data), 17β-estradiol-3-glucuronide (0.27%), and DHEA-sulfate (<0.01%). Interassay variation was 13.9% and the lowest point on the standard curve was 0.113 pg E_2/tube. All water blanks (n=8, 1–2 per assay) were non-detectable for E_2 (<0.113 pg).

Sex genotyping

Once plasma was collected from the centrifuged blood samples, some red blood cells were transferred to pieces of filter paper, which were stored at −20 °C in microcentrifuge tubes. We determined the sex of starling chicks via amplification of CHD genes using PCR as described previously (Griffiths et al., 1996; Chin et al., 2005). Briefly, DNA was isolated from red blood cells using Insta-gene matrix (Bio-Rad Laboratories, Hercules, California, Cat. No. 732-6030). DNA concentration was quantified with a spectrophotometer (Beckman Coulter DU 640). PCR amplification was performed using the P2 (5′-TCTGCATCGCTAAATCCTTT) and CW (5′-AGAAAT-CATTCCAGAAGTTCA) primer set. For positive controls, we used adult birds of known sex (n=5 of each sex). For negative controls, we used samples without DNA. PCR products were separated on a 3% agarose gel with ethidium bromide (70 V for 70 min). PCR products were visualized with a UV transilluminator.

Statistics

The effects of age, blood sampling site, and sex on DHEA and E_2 levels were assessed using three-factor analysis of variance (ANOVA), followed by *post hoc* multivariate analysis of variance (MANOVA) tests. We also calculated the difference between brachial and jugular steroid levels. This was accomplished by subtracting the average brachial DHEA or E_2 level (for a given age and sex) from each individual jugular DHEA or E_2 level, yielding a "difference score." The effects of age and sex on these difference scores were assessed using two-factor ANOVA tests, followed by *post hoc* MANOVA tests. If necessary, data were transformed by logarithm (base 10) transformation prior to ANOVA. If log-transformed data did not meet the assumptions of the ANOVA, they were analyzed using Welch's t-tests. Welch's t-tests, unlike Student's t-tests, do not assume homogeneity of variance (Welch, 1938). Plasma samples with non-detectable steroid levels were assigned the assay detection limit (2 pg for DHEA RIA, 0.113 pg for E_2 RIA). All tests were performed using SPSS 15.0. Statistical significance was set at $p<0.05$. Results are presented as mean±standard error of the mean.

Results

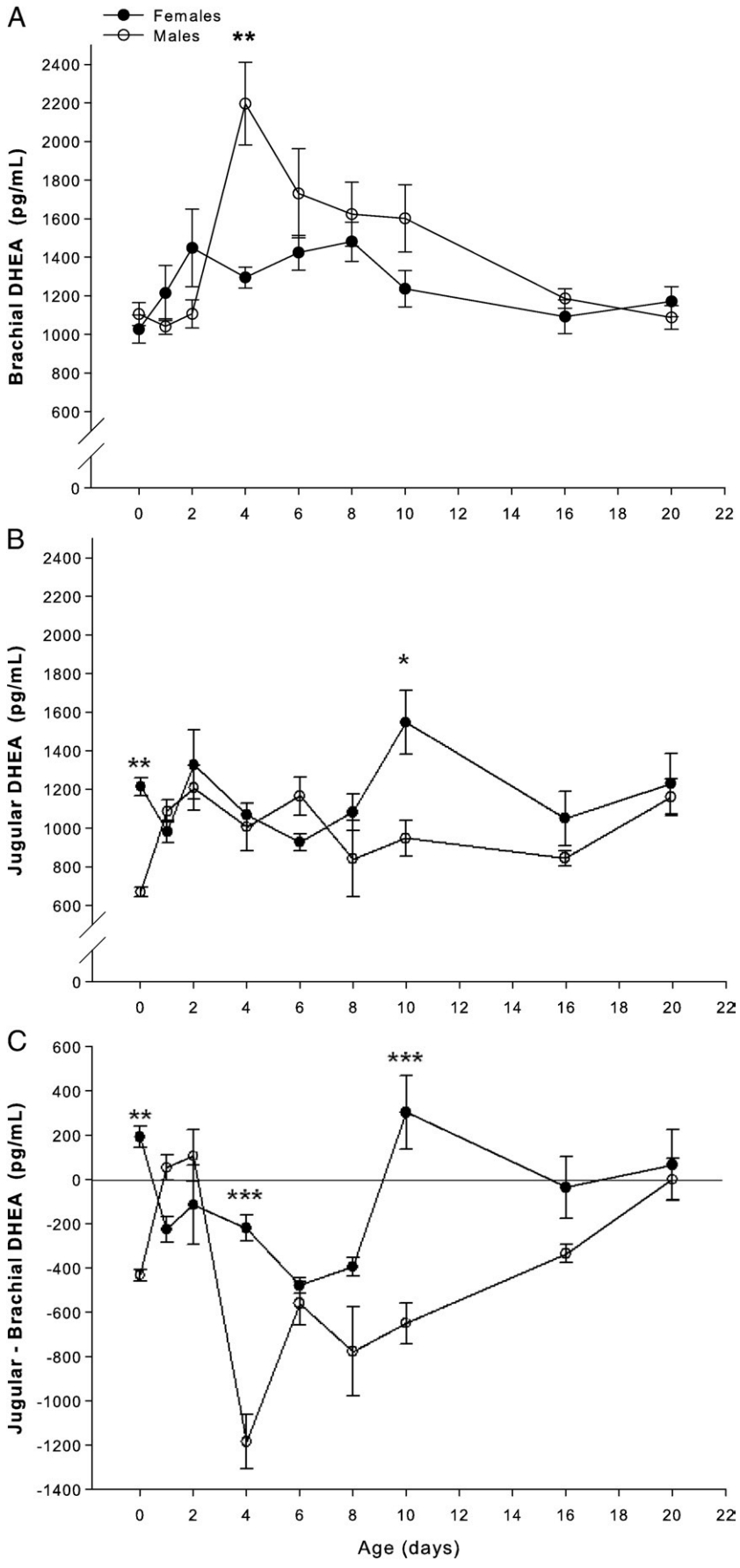
Body mass

Body mass increased across development (Fig. 2). Male starlings hatched at 6.38±0.26 g and fledged at 73.61±1.09 g, and female starlings hatched at 6.02±0.30 g and fledged at 70.81±1.28 g. For both sexes, body mass increased linearly from P0 to P10.

Plasma DHEA levels

DHEA was detected in all brachial (Fig. 3A) and jugular (Fig. 3B) plasma samples. The three-factor ANOVA indicated that there were significant main effects of age ($F_{8,303}$=3.81; $p<0.001$) and blood

Fig. 3. Plasma DHEA levels in nestling European starlings. (A) DHEA levels in brachial plasma. (B) DHEA levels in jugular plasma. (C) Differences between brachial and jugular DHEA concentrations (jugular–brachial levels). Negative "difference scores" indicate that DHEA levels are lower in jugular plasma than brachial plasma. Positive difference scores indicate that DHEA levels are higher in jugular plasma than brachial plasma. Juvenile starlings were sampled from hatch (day 0) to fledging (day 20). *$p<0.05$, **$p<0.01$, ***$p<0.001$ (males vs. females).


A
Females
Males
**
Brachial DHEA (pg/mL)
2400
2200
2000
1800
1600
1400
1200
1000
800
600
0
0 2 4 6 8 10 12 14 16 18 20 22
B
Jugular DHEA (pg/mL)
**
*
C
Jugular - Brachial DHEA (pg/mL)
**
***
***
600
400
200
0
-200
-400
-600
-800
-1000
-1200
-1400
Age (days)

sampling site ($F_{1,303}$=24.29; p<0.001), but not sex ($F_{1,303}$=0.033; p=0.857). Moreover, the three-way interaction (age×blood sampling site×sex) was significant ($F_{8,303}$=2.21; p=0.027). The three-way interaction was examined further with *post hoc* MANOVA tests. These tests looked for sex differences at each of the different ages for brachial and jugular plasma. For brachial DHEA, levels were highest on P4 in males (Fig. 3A). Importantly, brachial DHEA levels were significantly higher in males than females on P4 ($F_{1,303}$=9.68; p=0.002) (Fig. 3A). For jugular DHEA, levels were highest on P10 in females (Fig. 3B). In contrast to brachial DHEA, jugular DHEA levels were significantly higher in females than males on P0 ($F_{1,303}$=10.36; p=0.001) and P10 ($F_{1,303}$=5.83; p=0.016) (Fig. 3B).

To compare DHEA levels in brachial and jugular veins, the average brachial DHEA level (for a given age and sex) was subtracted from each individual jugular DHEA level (Fig. 3C). This yielded a "difference score," and negative difference scores indicate that brachial DHEA levels are higher than jugular DHEA levels. There were significant main effects of age ($F_{8,177}$=6.682; p<0.001) and sex ($F_{1,177}$=20.973; p<0.001) on DHEA difference scores. There was also a significant interaction between age and sex ($F_{8,177}$=5.122; p<0.001). *Post hoc* MANOVA analysis revealed that males had significantly lower DHEA difference scores than females on P0 ($F_{1,177}$=9.96; p=0.002), P4 ($F_{1,177}$=23.68; p<0.001), and P10 ($F_{1,177}$=21.51; p<0.001) (Fig. 3C). These data suggest that neural metabolism of circulating DHEA is greater in males than females on P0, P4, and P10. Overall, the DHEA difference scores were negative in males at most ages, suggesting that the male brain metabolizes and/or takes up circulating DHEA. Interestingly, DHEA difference scores were positive specifically in P0 and P10 females, suggesting that the female brain secretes DHEA into the general circulation at these ages.

Plasma E_2 levels

Plasma E_2 levels were ~100 fold lower than plasma DHEA levels (Figs. 4A,B). E_2 was detectable in 42% of brachial plasma samples and, surprisingly, only 62% of jugular plasma samples (Table 2). In general, plasma E_2 levels were elevated in both sexes during the first week post-hatch. Even after data transformation, the E_2 data failed to meet the homogeneity of variance assumption for the ANOVA, so we analyzed E_2 data using Welch's *t*-tests to examine sex differences at each age. Welch's *t*-tests do not assume homogeneity of variance (Welch 1938). For brachial E_2, levels were significantly higher in males than females on P6 (t (19)=2.44; p=0.025) (Fig. 4A). Unexpectedly, jugular E_2 levels showed no sex difference at any age (Fig. 4B).

To compare E_2 levels in brachial and jugular veins, the average brachial E_2 level (for a given age and sex) was subtracted from each individual jugular E_2 level (Fig. 4C). This yielded a "difference score" and negative difference scores indicate that brachial E_2 levels are higher than jugular E_2 levels. Even after data transformation, these data did not meet the homogeneity of variance assumption for the ANOVA and were analyzed using Welch's *t*-tests to determine sex differences at each age. Significant sex differences were found at P4 (t (14)=−3.02; p=0.009), P6 (t (28)=−2.99; p=0.006) and P8 (t (28)=−8.65; p<0.001) (Fig. 4C). These data suggest that neural metabolism and/or uptake of circulating E_2 is greater in males than females on P4, P6 and P8. Overall, the E_2 difference scores were negative in males at most ages, suggesting that the male brain metabolizes and/or takes up circulating E_2. E_2 difference scores were positive in P2 males and in P2, P4, P6 and P16 females, suggesting that the brain secretes E_2 into the general circulation in these animals.

Correlations between DHEA and E_2

In brachial plasma, DHEA and E_2 levels were significantly positively correlated (r=0.60, p<0.001). Interestingly, a higher correlation was found in males (r=0.66, p<0.001) than females (r=0.33, p=0.004).

In jugular plasma, DHEA and E_2 levels were also significantly positively correlated (r=0.27, p<0.001). Again, a higher correlation was found in males (r=0.34, p<0.001) than females (r=0.20, p>0.05). Overall, the correlation was lower in jugular plasma than brachial plasma.

Discussion

This is the first study to (1) examine circulating DHEA levels in developing songbirds and (2) directly compare endogenous DHEA and E_2 levels in the brachial and jugular veins. Moreover, this is one of the very few studies to examine hormone levels during development in wild songbirds (Silverin and Sharp, 1996; Williams et al., 1987). Importantly, little is known about DHEA during development in animals (Tagawa et al., 2005). Interestingly, brachial DHEA levels were higher in males than females on P4, within the critical period for sexual differentiation of the song system. In contrast, jugular DHEA levels were higher in females than males on P0 and P10. Brachial E_2 levels were higher in males than females on P6, and surprisingly, jugular E_2 levels showed no sex differences. DHEA and E_2 "difference scores" compared brachial and jugular steroid levels, and these data suggest that the developing female brain is more likely to secrete DHEA and E_2 into the general circulation, whereas the developing male brain metabolizes or sequesters circulating DHEA and E_2 to a greater extent.

Plasma DHEA levels

In brachial plasma, males exhibited higher levels of DHEA than females on P4. This timing corresponds to the timing of elevated jugular E_2 levels in male zebra finches (Hutchison et al., 1984). In addition, this peak in systemic DHEA occurs during the first week of life, the likely critical period for sexual differentiation of the song nuclei (Adkins-Regan et al., 1994). Moreover, these data suggest that there is a prominent peripheral source of DHEA. Two possible sources, the gonads and adrenals, both possess the steroidogenic enzymes to synthesize DHEA in developing zebra finches (Freking et al., 2000). Future studies will measure DHEA and steroidogenic enzymes in the gonads and adrenals of developing starlings.

In jugular plasma, the opposite sex difference was observed at P0 and P10, with females having higher jugular DHEA levels than males. These data suggest that male brains metabolize and/or sequester systemic DHEA to a greater extent than female brains at these ages. Alternatively, female brains may be secreting DHEA to a greater extent than male brains. The jugular DHEA data suggest sex differences in steroidogenic enzyme activities in the developing songbird brain. In adult zebra finches, there is a sex difference in 3β-hydroxysteroid dehydrogenase/isomerase (3β-HSD), the enzyme converts DHEA to androstenedione (Soma et al., 2004) (Fig. 1). There may be a sex difference in brain 3β-HSD during development (M>F), resulting in differential neural metabolism of systemic DHEA. A testable hypothesis is that genes encoding enzymes for DHEA metabolism are on the Z chromosome and expressed at higher levels in the brains of males (ZZ) than females (ZW).

To compare DHEA levels in the brachial and jugular plasma, DHEA "difference scores" were calculated. DHEA difference scores were

Fig. 4. Plasma E_2 levels in nestling European starlings. (A) E_2 levels in brachial plasma. (B) E_2 levels in jugular plasma. (C) Differences between brachial and jugular E_2 concentrations (jugular–brachial levels). Negative "difference scores" indicate that E_2 levels are lower in jugular plasma than brachial plasma. Positive difference scores indicate that E_2 levels are higher in jugular plasma than brachial plasma. Juvenile starlings were sampled from hatch (day 0) to fledging (day 20). The dashed line in (A) and (B) indicates the detection limit of the assay. *p<0.05, **p<0.01, ***p<0.001 (males vs. females).

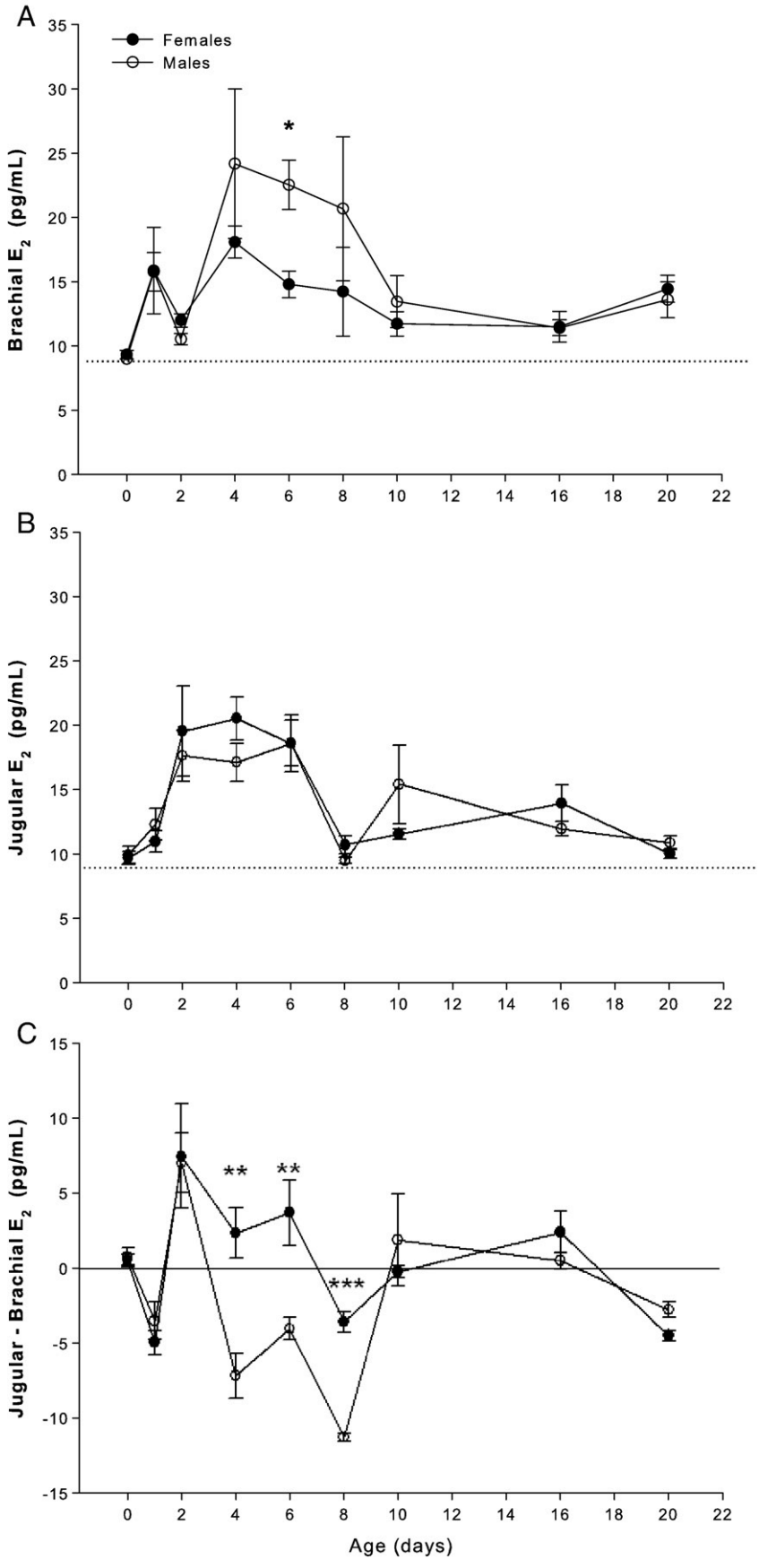

A
Females
Males
Brachial E_2 (pg/mL)
*
B
Jugular E_2 (pg/mL)
C
Jugular - Brachial E_2 (pg/mL)
**
**
***
Age (days)

Table 2
Percent of plasma samples with detectable E_2 in nestling European starlings

Age	Males		Females	
	Brachial: % detectable	Jugular: % detectable	Brachial: % detectable	Jugular: % detectable
P0	0	14	25	14
P1	50	40	80	27
P2	75	66	100	71
P4	100	88	100	100
P6	100	100	54	71
P8	75	33	25	38
P10	43	43	50	88
P16	60	54	33	71
P20	56	40	89	43

Note: Detection limit is 0.113 pg E_2/tube.

lower in males than females on P0, P4, and P10, again suggesting a sex difference in metabolism or uptake of DHEA by the brain. Furthermore, DHEA difference scores were often negative, particularly in males, indicating that jugular DHEA levels are lower than brachial DHEA levels. In contrast, in P0 and P10 females, DHEA difference scores were positive, suggesting that in these animals, the brain may secrete DHEA into the general circulation. Brain DHEA levels have been measured in developing chickens (Migues et al., 2002), and future studies will measure DHEA and DHEA-metabolizing enzymes, such as 3β-HSD and 17β-HSD, in developing starling brain.

Plasma E_2 levels

In brachial plasma, E_2 levels were generally low, and not all samples had detectable levels of E_2, despite the very high sensitivity of the assay. Interestingly, brachial E_2 levels were higher in males than females on P6, during the likely critical period for sexual differentiation of the song system. During this critical period, exogenous E_2 partially masculinizes the female song system in zebra finches and European starlings (Gurney and Konishi, 1980; Casto and Ball, 1996). The source of systemic E_2 in females is likely the ovary, which expresses high levels of aromatase during development (Schlinger and Arnold, 1992; Cam and Schlinger, 1998; Freking et al., 2000). The source for systemic E_2 in males is less clear. In zebra finches, the male adrenals and testes express little or no aromatase during development (Schlinger and Arnold, 1992; Cam and Schlinger, 1998; Freking et al., 2000). The male brain is also a possibility and expresses high levels of aromatase during development (Schlinger and Arnold, 1992; Cam and Schlinger, 1998). However, jugular E_2 levels were generally low (see below), arguing against this idea.

In jugular plasma, surprisingly, E_2 levels were also low, and no sex difference was detected at any age. Overall, jugular E_2 levels were high between P2 and P6. Our data from starlings do not correspond with E_2 data from zebra finches (Hutchison et al., 1984); this study reported very high jugular E_2 levels in males during the first week post-hatch. This discrepancy may be due to differences in E_2 antibody specificity or a species difference.

E_2 difference scores were lower in males than females on P4, P6, and P8, suggesting a sex difference in metabolism or uptake of E_2 by the brain during the critical period (as with DHEA). Unexpectedly, E_2 difference scores were negative in males from P4 to P8, indicating that jugular E_2 levels are lower than brachial E_2 levels. In contrast, E_2 difference scores were positive in males and females at P2, suggesting that the brain secretes E_2 into the general circulation at this age. Similarly, in adult male zebra finches and brown-headed cowbirds, after a peripheral injection of radiolabeled androgen, levels of radiolabeled estrogens are higher in the jugular plasma than carotid plasma (Schlinger and Arnold, 1991; Schlinger and Arnold, 1993; Saldanha and Schlinger, 1997).

Low levels of jugular E_2 and negative E_2 difference scores in males during the critical period were unexpected because (1) E_2 has been detected at high levels in the jugular plasma of developing male songbirds (Hutchison et al., 1984; Silverin and Sharp, 1996); (2) aromatase is highly expressed in the developing male zebra finch brain (Schlinger and Arnold, 1992); (3) brain slice cultures from developing male zebra finches secrete high amounts of E_2 into the medium (Holloway and Clayton, 2001); and (4) the steroidogenic enzymes necessary to synthesize E_2 from cholesterol are all expressed in the developing zebra finch brain (London et al., 2006). Moreover, several lines of evidence suggest that E_2 plays an important role in the masculinization of the song system (Gurney and Konishi, 1980; Adkins-Regan et al., 1994; Casto and Ball, 1996).

The present results suggest 2 possibilities: (1) E_2 is synthesized in the developing songbird brain but is rapidly metabolized or (2) E_2 is not the active estrogen during development. Regarding the first hypothesis, it is possible that E_2 is synthesized in specific brain regions, acts via estrogen receptors, and is then rapidly metabolized. Estrogens are inactivated in the periphery (e.g., liver) and also the brain via hydroxylation, glucuronidation, sulfonation, or *O*-methylation (Balthazart and Ball, 2006; Cornil et al., 2006). ^{3}H-estrogens are highly metabolized by the developing zebra finch brain, although the identity of the metabolite(s) remains unclear (Schlinger et al., 1994). Importantly, our results suggest that neural E_2 metabolism may be greater in males than females from P4 to P8. Regarding the second hypothesis, it is possible that 17β-E_2 is not the active estrogen in the developing brain. In developing mice, evidence suggests that 17α-E_2 levels are higher than 17β-E_2 levels in the brain and that 17α-E_2 is an active estrogen in the brain (Toran-Allerand et al., 2005). Future studies should measure 17α-E_2 in the songbird brain. Also, estrone (E_1) or estriol (E_3) could be the active estrogen during development. Plasma E_1 levels are higher than plasma E_2 levels in developing zebra finches (Schlinger and Arnold, 1992). However, preliminary data suggest that plasma and brain E_1 and E_3 levels are low in juvenile starlings (A. Shah and K. Soma, unpublished results). Androstenediol is another possibility (Jellinck et al., 2007).

Relationship between DHEA and E_2

Plasma DHEA and E_2 levels were positively correlated. Qualitatively, this correlation appeared (1) stronger in brachial plasma than jugular plasma and (2) stronger in males than females. If brain metabolism, uptake or secretion differs between DHEA and E_2, this might account for the lower correlation in jugular plasma. The stronger correlation between plasma DHEA and E_2 in males supports the idea that the males metabolize DHEA into E_2 to a greater extent than females. We were unable to examine the correlation between brachial DHEA and jugular E_2 levels in males, although this would be of great interest, because these levels were not measured in the same subjects.

Local versus systemic steroid signals during development

DHEA generally has been thought to be an inactive prohormone, requiring local conversion to active sex steroids within target tissues to have effects on the brain and other organs (Labrie et al., 2001; Widstrom and Dillon, 2004; Schlinger et al., 2008). In the presence of appropriate steroidogenic enzymes, DHEA can be converted to androgens and estrogens, which bind to androgen or estrogen receptors. Alternatively, some data suggests that DHEA can bind directly to androgen and/or estrogen receptors, independent of its metabolism to sex steroids (Lu et al., 2003; Chen et al., 2005b).

Overall, the present results are consistent with the hypothesis that systemic DHEA from a peripheral source is locally converted within the brain to E_2, which is rapidly metabolized. The present data do not rule out the hypothesis that E_2 is synthesized from cholesterol entirely

within the brain, precluding the need for circulating DHEA (Schlinger et al., 2001). These neuroendocrine mechanisms may avoid the "costs" of high circulating T (Wingfield et al., 2001; Soma, 2006) in developing males. Plasma T levels in juvenile starlings (150–600 pg/mL) (Williams et al., 1987) are far lower than plasma DHEA levels (this study). High circulating levels of DHEA, rather than T, during development may be a physiological strategy to avoid the negative impacts of high circulating T, as has been suggested for wintering adult male songbirds (Soma and Wingfield, 2001; Soma, 2006). High circulating T can suppress immune function (Owen-Ashley et al., 2004; Fargallo et al., 2007). High circulating T also suppresses body growth in nestlings, which is a major cost during development (Groothuis and Ros, 2005; Fargallo et al., 2007). Circulating DHEA may only affect tissues that express the appropriate enzymes, conferring high spatial specificity of steroid action.

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