3β-HSD expression in the CNS of a Manakin and Finch

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Abstract

The prohormone, dehydroepiandrosterone (DHEA) circulates in vertebrate blood with the potential for actions on target tissues including the central nervous system (CNS). Many actions of DHEA require its conversion into more active products, some of which are catalyzed by the enzyme 3β-hydroxysteroid-dehydrogenase/isomerase (3β-HSD). Studies of birds show both expression and activity of 3β-HSD in brain and its importance in regulating social behavior. In oscine songbirds, 3β-HSD is expressed at reasonably high levels in brain, possibly linked to their complex neural circuitry controlling song. Studies also indicate that circulating DHEA may serve as the substrate for neural 3β-HSD to produce active steroids that activate behavior during non-breeding seasons. In the golden-collared manakin (*Manacus vitellinus*), a sub-oscine bird, low levels of courtship behavior are displayed by males when circulating testosterone levels are basal. Therefore, we asked whether DHEA circulates in blood of manakins and whether the brain expresses 3β-HSD mRNA. In order to test that the manakin spinal cord is a target of androgens due to its importance in regulating acrobatic movements, we also examined expression of this enzyme in the manakin spinal cord. For comparison, we examined expression levels with those of an oscine songbird, the zebra finch (*Taeniopygia guttata*), a species in which brain, but not spinal cord, 3β-HSD has been well studied. DHEA was detected in manakin blood at levels similar to that seen in other species. As described previously, 3β-HSD was expressed in all zebra finch brain regions examined. By contrast, expression of 3β-HSD was only detected in the manakin hypothalamus where levels were greater than zebra finches. In spinal cord, 3β-HSD was detected in some but not all regions in both species. These data point to species differences and indicate that manakins have the substrate and neural machinery to convert circulating DHEA into potentially active androgens and/or estrogens.

48 Keywords: androgen, brain, courtship, DHEA, enzyme, spinal cord steroid

49

50 **1. Introduction**

51 Steroid hormones are critical for the expression of adaptive phenotypes in vertebrates living in a
52 variety of social and/ or biotic environments (Nelson, 2011). Often, the underlying mechanisms
53 are difficult to discern by only measuring circulating hormone levels, because steroid levels in
54 blood are not always congruent with those found in tissues (Pradhan et al., 2015; Schmidt et al.,
55 2008), and some circulating hormones require conversion into more active metabolites by the
56 locally expressed steroid-metabolic enzymes (London et al., 2009; Vanson et al., 1996). Among
57 these enzymes, 3β-hydroxysteroid dehydrogenase (3β-HSD) is critically positioned in the
58 steroidogenic pathway and is well studied in the gonads and adrenals (Freking et al., 2000;
59 Schlinger et al., 2008). Nevertheless, 3β-HSD is also expressed in other vertebrate tissues
60 including liver, heart, aorta, kidney, spinal cord and brain (Coirini et al., 2002; Nakamura et al.,
61 2005; Payne and Hales, 2004; Zhao et al., 1991; Zhao et al., 1990) where its function is only
62 recently being fully appreciated. This enzyme is essential for the conversion of pregnenolone to
63 progesterone and of dehydroepiandrosterone (DHEA) to androstenedione, which, in turn, is the
64 substrate for the production of the more potent androgens, testosterone (T) and perhaps also 5α-
65 dihydrotestosterone, that binds strongly to the androgen receptor.

66 Studies of birds show both expression and activity of 3β-HSD in brain (Tsutsui, 2011;
67 Vanson et al., 1996) as well as possible neurobehavioral functions. For example, in the oscine
68 songbird brain, 3β-HSD functions coordinately with the estrogen synthetic enzyme aromatase to
69 convert DHEA into estrogens (Pradhan et al., 2010a; Rohmann et al., 2007; Tam and Schlinger,
70 2007). These estrogens may then activate neural estrogen receptors to regulate social behaviors,

71 such as estrogen-dependent aggressive behavior expressed during the non-breeding season when
72 circulating testosterone is basal (Soma et al., 2000; Soma and Wingfield, 2001; Pradhan et al.,
73 2010).

74 Birds of the Order Passeriformes are separated into the oscine songbirds (such as the
75 zebra finch), that learn complex songs and possess a complex neural circuitry that underlies song
76 learning (Nottebohm et al., 1976), and the sub-oscines, species that lack complex song and most
77 neural structures comprising the oscine song system (Sibley and Ahlquist, 1985). Elevated
78 neural expression of 3β-HSD may be associated with the presence of the oscine neural song
79 system or it might be a general property of the Passeriform brain, a question that can be explored
80 by examination of RNA expression in different Passeriform species. In zebra finches, 3β-HSD
81 has been shown in organotypic brain slices and microdissected brain regions during both
82 development and adulthood (London et al., 2006; Tam and Schlinger, 2007), where it is
83 regulated by stress (Soma et al., 2004) as well as by 17β-estradiol (Pradhan et al., 2010a;
84 Pradhan et al., 2008). Thus, whereas 3β-HSD has been examined using multiple levels of
85 analysis in zebra finches, similar studies are lacking for a sub-oscine passerine bird.

86 Our laboratory has studied the neuroendocrine basis of behavior in a sub-oscine species,
87 the golden-collared manakin (*Manacus vitellinus*) of Panamanian rainforests. Males of this
88 species perform physically elaborate courtship displays daily over the course of the 6-7 month-
89 long reproductive season. These displays depend on androgens (Feng et al., 2010; Fusani et al.,
90 2007; Fuxjager et al., 2012b; Schlinger et al., 2013); nevertheless, even during the breeding
91 season, circulating T levels in males are extremely variable with some displaying males having
92 little or no measurable T levels in the blood (Day et al., 2007; Fusani et al., 2007). Interestingly,
93 juvenile males, as well as adult males during the nonbreeding season, exhibit low levels of

courtship even with low circulating levels of T (Day et al., 2007; Fusani et al., 2007). One mechanism that could explain these observations, is that DHEA circulates in manakin blood and functions as a substrate for 3β-HSD in brain to activate courtship behavior in breeding or non-breeding birds with low levels of circulating T. To address this question, we first asked if DHEA is present in manakin blood with greater levels in courting males as compared to females or non-courting (juvenile) males. Next, we asked whether 3β-HSD is expressed in the manakin central nervous system (CNS) to potentially utilize circulating DHEA substrate for the formation of more active androgens and/or estrogens. We used quantitative PCR to measure 3β-HSD mRNA expression in micro-dissected regions of the brain and spinal cord of adult males and female manakins. To evaluate potential differences between the sub-oscine manakin and an oscine songbird, we included adult male and female zebra finches in the expression analysis. CNS regions of interest were selected based on previous studies showing significant androgen and/or estrogen binding or receptor expression in manakins suggesting their possible function in activating and controlling male courtship displays (Fusani et al., 2014; Fuxjager et al., 2012b; Schultz and Schlinger, 1999).

2. Materials and Methods

2.1. Animals

All research was conducted with approval of appropriate governmental agencies and under the strict guidelines of the Animal Care and Use Committee at the University of California, Los Angeles (UCLA) and the Smithsonian Tropical Research Institute (STRI). Manakin blood (n=25) and tissue samples (n=12) were collected during the courtship season (February-April 2010, 2011) from forests in and around Gamboa, Panama. Reproductively active zebra finches

(n=24) were obtained from our UCLA colony.

2.2. Tissue Collection

Blood samples were collected in Panama from adult (n=14) and juvenile males (n=5) and adult females (n=6). Animals were captured using mist-nets and bled by venipuncture within 10 min of capture. Blood was kept at 4°C and then centrifuged at 1000 g within 3 h to yield on average 65 µl (30 –100 µl) plasma. Manakin brain tissues were collected immediately upon decapitation, dissected into the cerebellum (Cb), hypothalamus (Hyp), and left telencephalon (Tel), placed on dry ice and then stored either on dry ice or in a -80°C freezer at the Smithsonian Tropical Research Institute facilities in Panama City until shipped to UCLA; spinal cords were dissected into the cervical, thoracic and lumbosacral regions and placed in RNAlater solution. Appropriate aliquots were based off of weight of each sample and then refrigerated 2-8°C. All of the same brain tissues were collected from male (n=6) and female (n=6) zebra finches, with the exception that the whole Tel was collected. Spinal cords were collected from a separate group of male (n=6) and female (n=6) zebra finches. All tissues were frozen in dry ice immediately upon dissection and then stored at -80°C until assays. We have previously found no difference in RNA expression levels for zebra finch tissues placed in RNAlater or frozen immediately on dry ice (Fuxjager et al., 2015).

2.3. DHEA Measures

Concentration of DHEA was measured using a commercial kit (DSL 8900, Diagnostic Systems Laboratories, Webster, Texas, USA) with modifications as described previously (Granger et al., 1999) to increase assay sensitivity. This assay has been validated for a number of bird species

(Chin et al., 2008; Goodson et al., 2005; Newman et al., 2008a; Newman et al., 2008b). Briefly, plasma samples were extracted twice, each time using 3 mL dichloromethane (Newman et al., 2008b) using the freeze-decanting method, in which the water phase is snap-frozen on a mixture of ethanol and dry ice and the organic phase decanted (Canoine, 2001). Extracted samples were dried down and re-suspended in 220 µL PBS with 0.1% BSA and then assayed in duplicate. The initial sample volume was on average 53 µL. The detection limit was 128 pg / mL and the intra-assay coefficient of variation was <12%. We did not measure recovery in this study, though this method typically yields steroidal recoveries of 80-90% {Newman, 2008 #2257}.

2.4. RNA and PCR

Total RNA was extracted from tissue samples by TRIzol© Reagent (Invitrogen, Carlsbad,CA) and following the manufacturer's instructions. Tissues were homogenized in TRIzol© for ~40 s at medium/high speed with a standard stator homogenizer. Note that for RNA extractions, half the Tel (left) was used for manakin and the whole Tel was used for zebra finch. RNA concentration was measured with a Nanodrop System 1000 (Thermo Scientific, Wilmington, DE, USA), and integrity was assessed using gel electrophoresis. For both species, the 260/280 values had a range of 1.99-2.05 and the RNA concentrations, based on absorbance at 260 nm, had a range of 550-980 ng/ µL. There was no statistical difference in quality or quantity of RNA across species or CNS region. The volume of RNA that yielded 1000 ng/ mL was used for cDNA preparation. RNA samples were treated with DNAse (Promega, Madison, WI) and then reverse transcribed using Superscript Reverse Transcriptase II (Invitrogen) for 50 min at 42°C followed by 15 min at 70°C. To verify the presence of 3β-HSD transcripts in manakin and zebra finch tissues, resultant cDNA was used at a 1:10 dilution for PCR amplification.

We first performed RT-PCR using primers specific to zebra finches (London et al., 2006). For each species, we used a separate pool of brain tissue that included the regions of interest for this study. Following the reactions, the samples were assessed by gel electrophoresis and compared against a ladder. The samples that yielded a band at the appropriate weight were sequenced. Using this sequence, we used Primer 3.0 to design qPCR primers for both zebra finches and manakins. While the zebra finch primers worked reliably for both zebra finches and manakins, primers generated through the manakin specific sequences did not yield efficient qPCR reaction efficiencies and dissociation curves.

The following primers were validated by PCR to confirm specific amplification of 3β-HSD in zebra finch and golden-collared manakin for the brain/ spinal cord tissues: F, 5' – AGGGCGTACTCGCTCGTCATCC – 3' and R, 5' – TAGAGCACGGTCAGAGGCATGG – 3' (230 bp, T_m=63.9°C). The PCR reaction volume was 21 µL and contained the following: 0.38 mM deoxynucleotide triphosphate (mix), 0.4 µM forward and 0.4 µM reverse primer, 50 ng of respective sample cDNA, 0.06 ng DNA Taq Polymerase (Bioline, Randolph, MA), 2.5 µL KCl buffer, and 17.35 µl of sterile water. Reactions were run on a Thermocycler at 95°C for 5 min and then subjected to 38 cycles of 95°C for 30 s, 63.9°C for 30 s, 72°C for 1 min. Reactions were completed at 72°C for 10 min. PCR products for 3β-HSD were verified by gel electrophoresis to ensure that product size matched the expected base pair length. A sample of PCR amplified products from manakins was sequenced (Genewiz Inc., La Jolla, CA, USA) and blasted against the zebra finch genome confirming the identity as 3β-HSD. Identity of sequence for manakins was confirmed to be 99% similar to the zebra finch genome by BLAST analysis, with an E value of zero (http://www.ncbi.nlm.nuh.gov/blast/).

187 **2.5. Quantitative PCR**

188 To determine the relative abundance of 3β-HSD in manakin and zebra finch tissues (cDNA
189 dilution 1:5), we performed quantitative PCR (qPCR) using an ABI 7300-96 well sequence
190 detection system with SYBR Green PCR master Mix (Applied Biosystems Inc., Foster City,
191 CA). Based on the PCR results, Primer 3.0 was used to design qPCR primers with a low GC
192 count and spanning exons. The following qPCR primer pair was used, each at a concentration of
193 18 µM, and was verified for both zebra finches and manakins: F, 5' –
194 AGGGCGTACTCGCTCGTCATCC – 3' and R, 5' – TAGAGCACGGTCAGAGGCATGG –
195 3'. The reference gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was based on
196 annotated the zebra finch sequence and 0.3 µM for each, forward and reverse primer, was used
197 for all samples (F: 5' – TGACCTGCCGTCTGGAAAA, R: 5' –
198 CCATCAGCAGCAGCCTTCA). GAPDH is a frequently used as an internal control gene
199 because its expression is unaffected by steroid treatment (McGraw et al., 2005). GAPDH is also
200 relatively stable reference gene for zebra finch brain and white-throated sparrow (*Zonotrichia*
201 *albicollis*) tissues (Zinzow-Kramer et al., 2014). While the GAPDH primer sequence was based
202 on zebra finches, previous studies from our lab have shown that its properties do not detectibly
203 differ in primer binding and reaction efficiencies among zebra finches and manakins (Bustin et
204 al., 2009; Feng et al., 2010). For each species, there was no difference in Ct values of GAPDH
205 among brain or spinal cord regions ($p>0.05$). Total volume for each reaction was 25 µL, with
206 2.5 µl diluted cDNA at a dilution of 1:10 (280 ng RNA; 1-1.5 µL cDNA was diluted
207 appropriately) and 22.5 µL SYBR green. All qPCR reactions were carried out as follows: 50°C
208 for 2 min, 95°C for 10 min, and 40 cycles at 95°C for 15 sec/60°C for 1 min, with assay
209 completed a 95°C for 15 sec, 60°C for 30 sec, and 95°C for 15 sec (with a dissociation stage at

the end of each reaction). Dissociation curves of the qPCR products were assessed to ensure absence of DNA contamination. All samples were run in duplicate for both genes. Standard curves were determined for each reaction, along with correlated coefficients and known concentration of cDNA, to generate slopes that could calculate amplification efficiency for each primer reaction. For each qPCR assay, Ct values were generated and relative expression against GAPDH was determined; ∂CT was calculated using the following formula: [1000 X ($2^{-[CT\ gene\ of\ interest - CT\ GAPDH]}$)].

2.6. Data Analysis

DHEA levels were analyzed across adult males, adult females and juvenile males using one-way ANOVA. For all qPCR reactions, we calculated the reaction efficiencies using the standard curves of GAPDH and 3β-HSD. For GAPDH, all qPCR reaction efficiencies met the range criterion of 90-115%, Ct values were detectible, and the dissociation curves were of good quality. For 3β-HSD, however, Ct values for some samples were quite low producing Ct values greater than 35 or undetectable, values we deemed likely unreliable and which we describe as not reaching criterion. In addition, for some CNS regions of interest, the reaction efficiencies for 3β-HSD were beyond the acceptable range.

In order to use all the data reliably, we used two different sets of analyses. In the first case, we used the Wilcoxon Signed Rank Test to non-parametrically evaluate the proportion of individual manakins and zebra finches across brain and spinal cord regions that had a Ct value <35 for 3β-HSD (i.e. those that met criterion; Table 1).

In the second case, we parametrically analyzed only those regions where >50% of the samples met criterion. However, we used the actual Ct values in the analyses and we assigned a

Ct value of 40 (the detection limit of the assay) for those values that were undetectable. We then computed the ∂CT.

To assess regional differences in 3β-HSD mRNA expression (within brain and spinal cord across species and sex), ∂Ct values were analyzed by two-way ANOVA. Because assumptions regarding normality of distribution and equality of variances were not met data, were log transformed. We performed three separate sets of these analyses. First, in the brain, only the hypothalamus, and in the spinal cord, only the thoracic region, reached criteria for both species (Table 1). Hence, for each of these regions, we used Species (zebra finch or manakin) and Sex as between subject factors. Second, all zebra finch brain regions reached criterion, and so we used Sex (male or female) and Region (Cb, Hyp, and Tel) as between-subject factors. Third, in the spinal cords of zebra finches, the thoracic and lumbar reached criterion and for manakins the thoracic and cervical regions reached criterion. Hence for each species, we used Region (zebra finch: Thoracic versus Lumbar, manakin: Cervical versus Thoracic) and Sex as between-subject factors. There were no significant effects of Sex (no main effect and no interaction) and hence male and female subjects were pooled and analyzed with a Tukey Multiple comparison test. Due to relatively small sample sizes for each sex, our data should be interpreted with caution. All data were analyzed using GraphPad Prism 7.0 for Macintosh. Significance was accepted at $\alpha < 0.05$, and all data are shown as mean ± SEM.

3. Results

3.1. Plasma DHEA levels

DHEA was detected in plasma of all 25 individuals examined with most falling between 1-2 ng/ml plasma (Figure 1). One-way ANOVA ($F_{2,23}$= 1.19, p=0.33) revealed no significant differences across groups.

3.2. 3β-HSD in brain regions

There were overall differences in 3β-HSD expression across all brain regions of manakins and zebra finches through the Wilcoxon Signed Rank Test of number of individuals reaching the criteria for qPCR reactions (p= 0.0312). While 3β-HSD was expressed in all three brain regions in zebra finches, it was expressed reliably only in the Hyp of manakins (also see Table 1). Therefore we restricted our between species comparison to the Hyp (Figure 2). Manakins had significantly higher Hyp 3β-HSD mRNA expression compared to zebra finches ($F_{1,20}$= 22.39, p=0.0001), but there was no main effect of Sex ($F_{1,20}$= 1.45, p=0.24) and no Sex*Species interaction ($F_{1,20}$= 0.82, p= 0.38).

In the zebra finch brain, there were significant differences across the regions (Figure 3, $F_{2,33}$ = 15.16, p< 0.0001), such that the mRNA expression of 3β-HSD was higher in the Cb than the Hyp (q= 7.58, p< 0.0001) and Tel (q= 2.23, p= 0.27).

3.3. 3β-HSD in spinal cord regions

There were overall differences in 3β-HSD expression across all spinal cord regions of manakins and zebra finches through the Wilcoxon Signed Rank Test of number of individuals reaching the criteria for qPCR reactions (p= 0.0312). 3β-HSD was detected reliably in manakin cervical and thoracic regions and zebra finch thoracic and lumbar regions (Table 1; Figure 4A). A subsequent analysis across species for the thoracic region only showed no significant main effects of Species

277 ($F_{1,15}$= 0.23, p= 0.64) and Sex ($F_{1,15}$= 1.56, p= 0.23), or significant Species*Sex interactions
278 ($F_{1,15}$ = 0.63, p= 0.44).
279 When species were analyzed separately (cervical and thoracic regions in manakins;
280 thoracic and lumbar in zebra finches, Figure 4B, C), we found no significant region, sex or
281 region* sex interactions for either manakins (Region, $F_{1,13}$= 0.98, p= 0.34; Sex, $F_{1,16}$= 0.04, p=
282 0.85; Region*Sex, $F_{1,16}$= 2.71, p= 0.12); or zebra finches, (Region, $F_{1,16}$= 0.63, p= 0.3; Sex,
283 $F_{1,16}$= 3.93, p= 0.06; Region*Sex, $F_{1,16}$= 1.09, p= 0.31).
284
285
286 **4. Discussion**
287 Through the data presented in this study, we expand our appreciation of the potential pro-
288 hormonal role of DHEA in the avian CNS by showing that 1) DHEA is found to circulate in
289 blood of a wild sub-oscine species, the golden-collared manakin, at levels generally similar to
290 that measured in other avian species; 2) that there are inter-species differences in the brain
291 expression of 3β-HSD mRNA 3) that 3β-HSD is expressed in the spinal cords of both
292 Passeriformes species studied; and 4) there are no sex differences in 3β-HSD expression in either
293 species. Species differences in the mRNA expression of 3β-HSD throughout the CNS points to
294 possible functional differences across species.
295
296 **4.1. Plasma DHEA in manakins**
297 We found that DHEA is readily detected in the plasma of adult male and female
298 manakins, as well as in juvenile males. These levels are similar to what has been reported for
299 other bird species (Newman et al., 2008b). We detected no significant sex difference, although

300 mean levels were somewhat more variable in females. These data support the idea that DHEA is
301 an important circulating hormone in these birds and adds sub-oscine species to the growing list
302 of birds in which circulating DHEA can be detected.
303 In many mammals and birds, the adrenals are the main source of DHEA and it is found
304 circulating at relatively high levels in both males and females (Schlinger et al., 2008). Moreover,
305 in humans, DHEA circulates at high levels in young adults and declines with age (Rainey et al.,
306 2002). Although no specific receptor for circulating DHEA has been identified (Widstrom and
307 Dillon, 2004), neural DHEA may serve as a substrate for the formation of active androgens
308 and/or estrogens that function via their specific receptor pathways (see discussion below). Such
309 actions may be especially important in non-reproductive conditions when DHEA in blood can
310 activate steroid-dependent circuits in the brain with little effect on other reproductive tissues
311 (Schlinger et al., 2008; Soma, 2006).
312 Circulating DHEA may also have actions fully independent of neural 3β-HSD. DHEA
313 could have direct or indirect effects on the HPG (Hypothalamic Pituitary Gonadal) Axis (Labrie
314 et al., 2005). DHEA could protect and regulate the immune/nerve functions within this avian
315 system (Veiga et al., 2003). There is also evidence that DHEA can protect the brain from some
316 deleterious actions of corticosteroids (Kalimi et al., 1994). While adrenalectomy to remove the
317 source of circulating DHEA is not feasible in these birds, future studies to block adrenal
318 androgen synthesis would be a useful next step to fully understand the mechanism of this
319 molecule's vast reach.
320
321 **4.2. 3β-HSD expression in the Brain**
322 3β-HSD has been identified in the brains of several vertebrates with significant attention

323 paid to the presence and function of this enzyme in the brains of birds. In songbirds, 3β-HSD is
324 expressed and is active in a region-specific manner (London et al., 2006; Pradhan et al., 2010b).
325 Interestingly, our data show that both manakins and zebra finches express 3β-HSD mRNA in the
326 Hyp. Moreover, we found a species difference, such that the manakins express ~8x higher levels
327 of 3β-HSD in this region. Thus, manakins have high levels of 3β-HSD concentrated in one brain
328 region. The Hyp regulates numerous behavioral and physiological systems, so these data are
329 intriguing evidence for a functionally important role for 3β-HSD in manakins.
330 While manakins express little if any 3β-HSD in the Cb and Tel, zebra finches have
331 reliably detectable expression in both brain regions, with highest levels in the Cb. Previous
332 studies have shown high levels of expression and activity of 3β-HSD and other steroidogenic
333 enzymes throughout the zebra finch brain (Freking et al., 2000; London et al., 2006; Soma et al.,
334 2004). This relatively high neural expression of steroidogenic enzymes in the oscine zebra finch
335 Tel is thought to be associated their complex song control neural circuitry (London et al., 2009).
336 The present data showing negligible expression of 3β-HSD in the sub-oscine manakin Tel lends
337 support for this hypothesis. Why zebra finches, but not manakins, express 3β-HSD in the Cb is
338 unknown, but may reflect the generalized increase in steroidogenic enzyme expression that
339 extends across the zebra finch CNS. Androgen receptors are expressed at relatively high levels
340 in the manakin arcopallium, located in the Tel, as well as in the midbrain nucleus
341 intercollicularis and in cerebellar Purkinje cells (Fusani et al., 2014). Thus, we predicted that
342 elevated 3β-HSD expression in these same regions of the male manakin brain to help activate
343 vocal and motor output of their courtship. However, our results suggest that DHEA metabolism
344 to more potent androgens likely does not act in these regions to promote courtship behavior. A
345 more focused examination of specific nuclei might have provided better resolution and revealed

346 some 3β-HSD expression in androgen-sensitive regions of the telencephalon and cerebellum, a
347 useful future experiment.
348 In the songbird brain, 3β-HSD can be coupled with the estrogen synthetic enzyme
349 aromatase to convert DHEA into estrogens (Soma et al., 2004; Tam and Schlinger, 2007; Vanson
350 et al., 1996). Estrogens promote some masculine reproductive behaviors in birds (Schlinger and
351 Brenowitz, 2009). Manakins express aromatase in the Hyp suggesting that estrogens might
352 activate some aspects of courtship in male manakins (Saldanha et al., 2000). The co-expression
353 of 3β-HSD in the Hyp supports a mechanism for DHEA to impact manakin behavior via an
354 estrogen-dependent pathway. Irrespective of the mechanism, the elevated expression of 3β-HSD
355 in the manakin Hyp points to a functional role in this species that may include the metabolism of
356 DHEA or other steroidal substrates.
357
358 **4.3. 3β-HSD expression in the spinal cord**
359 Given the motoric complexity of manakin courtship, as well as the significant expression of
360 spinal cord androgen receptors and androgen binding (Fuxjager et al., 2012a; Fuxjager et al.,
361 2013; Fuxjager et al., 2012b; Fuxjager et al., 2016), we expected to identify high levels of 3β-
362 HSD in the spinal cord. Although it is intriguing that 3β-HSD was expressed in selected regions
363 of both the manakin and zebra finch spinal cords, the distribution of mRNA expression we
364 observe lends little support for the idea that 3β-HSD is especially important in the manakin
365 spinal cord or is related to complex courtship. Importantly, 3β-HSD not only uses DHEA as a
366 substrate, but can also convert pregnenolone into progesterone, a conversion well studied in the
367 rodent spinal cord (Schumacher et al., 2004). Although we did not measure circulating
368 pregnenolone in this study, or examine steroidogenic enzymes upstream of 3β-HSD, it is

possible that progesterone is synthesized where we have identified 3β-HSD using local or circulating substrates (Do-Régo et al., 2009; Tsutsui, 2011; Vanson et al., 1996). In turn, progesterone or its functional metabolites, could exert an influence on neural spinal circuits in both manakins as well as zebra finches. Both 5α- and 5β-reductase are expressed in the oscine and sub-oscine spinal cord (Fuxjager et al., 2016) and could use progesterone to synthesize isoforms of allopregnanolone, potent modulators of GABA-A receptors (Carlisle et al., 1998). Together, these data indicate that more than one steroidogenic pathway and more than one localized region may require consideration in evaluating the role of 3β-HSD in the avian spinal cord.

4.4. Conclusions

In summary, circulating DHEA may function directly or indirectly to influence various tissues in Passeriform birds. The presence of 3β-HSD in some regions of the brain and spinal cord of male and female manakins and zebra finches, argues that 3β-HSD is a relatively conserved feature of the avian central nervous system, though more work is needed to ascertain what role this enzyme plays in avian neurobiology.

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Figure legends

Figure 1. Plasma DHEA levels in free-living golden collared manakins (adult males, n=14; adult females, n=6, and juvenile males, n=5).

Figure 2. Expression of 3β-HSD mRNA across brain of male (n=6) and female (n=6) zebra finches (ZF) and golden collared manakins (GCM). Expression in the hypothalamus was significantly greater in manakins compared to zebra finches.

Figure 3. Expression of 3β-HSD mRNA across zebra finches (ZF) brain, with both sexes combined (N=12). Expression in the cerebellum was significantly greater compared to both hypothalamus and telencephalon. **p<0.01; ***p<0.001

Figure 4. Expression of 3β-HSD mRNA across spinal cords of zebra finches (ZF) and golden collared manakins (GCM). There were no differences in (A) Expression across species and across sexes in the thoracic region (B) Expression in the cervical versus thoracic region of GCM or (B) Expression in the thoracic and lumbar region of ZF. For ZF, n=5 males and n=5 females; for GCM, n=4 males and n=5 females ***p<0.001.

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552

Figure 1

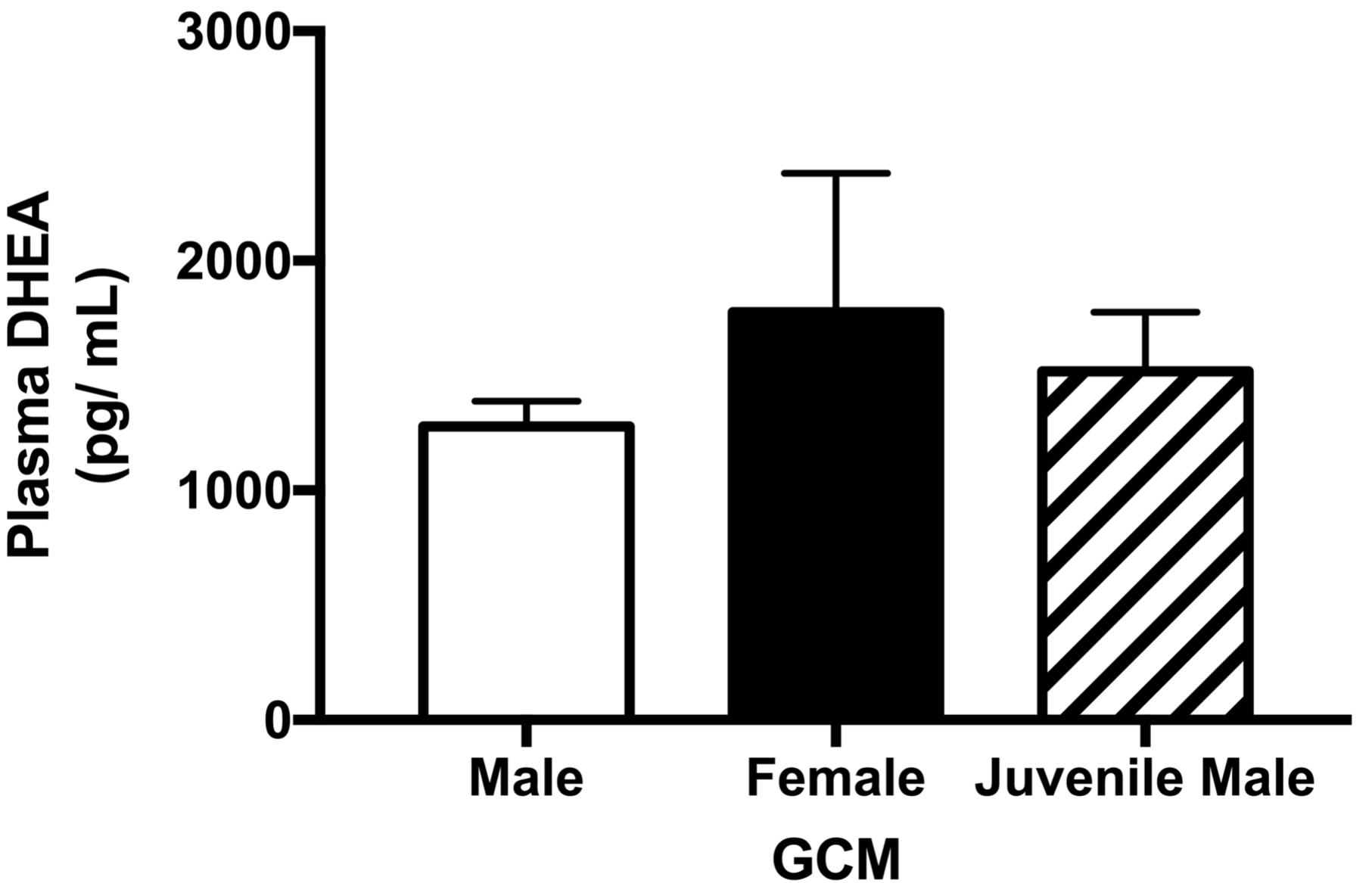
3000
2000
1000
0
Plasma DHEA
(pg/ mL)
Male
Female
Juvenile Male
GCM

Figure 2

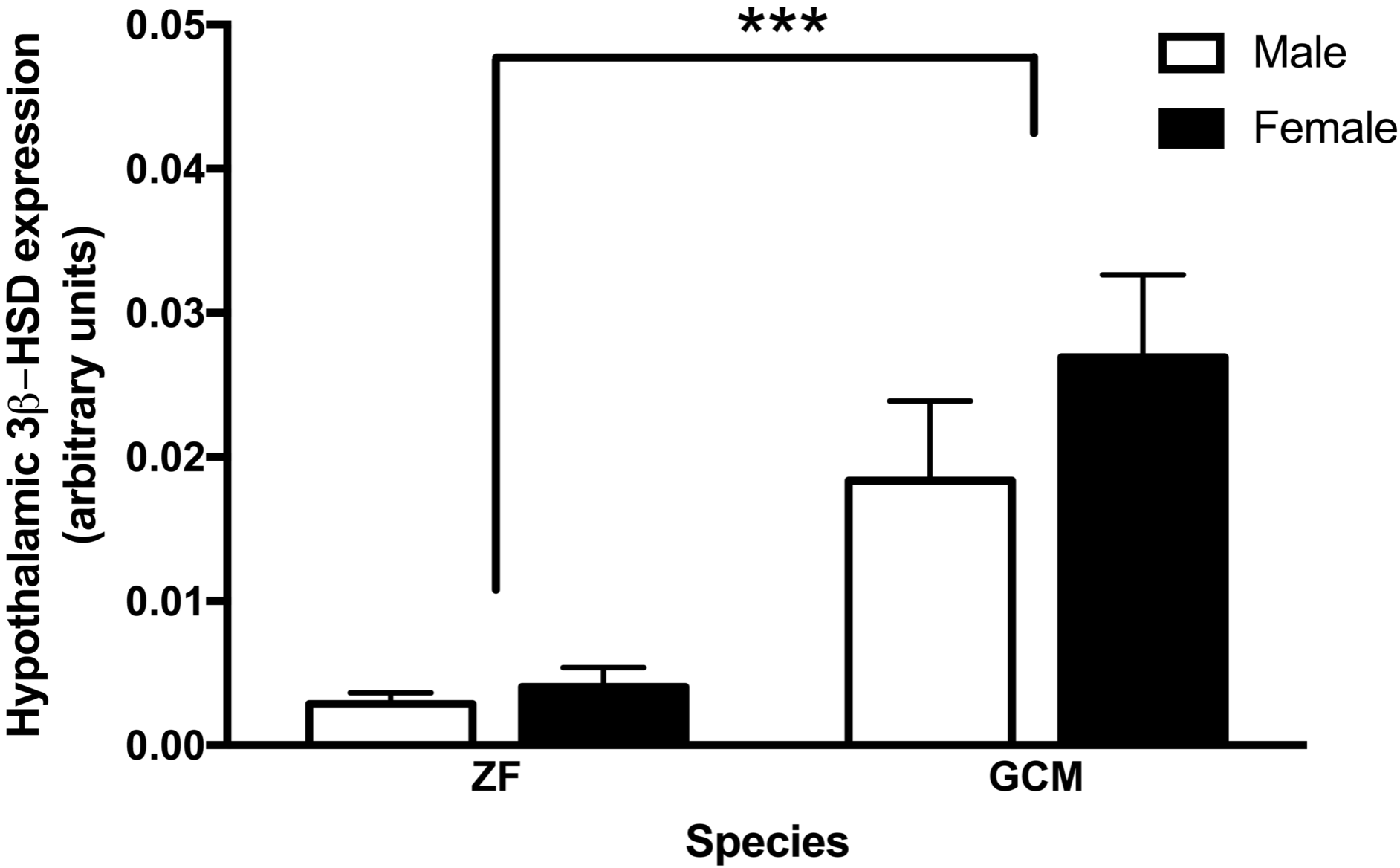
***
Male
Female
0.05
0.04
0.03
0.02
0.01
0.00
Hypothalamic 3β–HSD expression
(arbitrary units)
ZF
GCM
Species

Figure 3

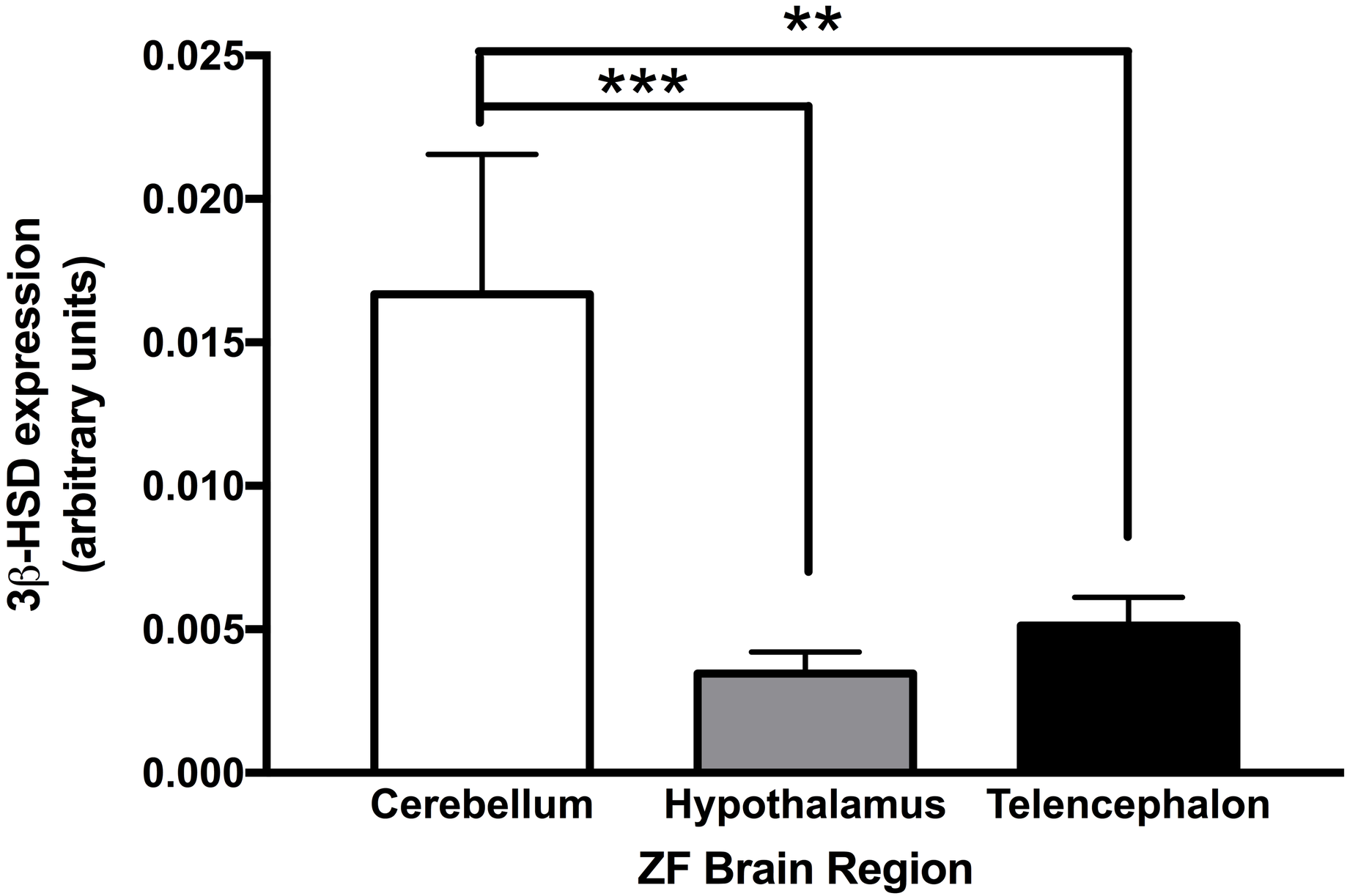
**
***
0.025
0.020
0.015
0.010
0.005
0.000
3β-HSD expression
(arbitrary units)
Cerebellum
Hypothalamus
Telencephalon
ZF Brain Region

Figure 4

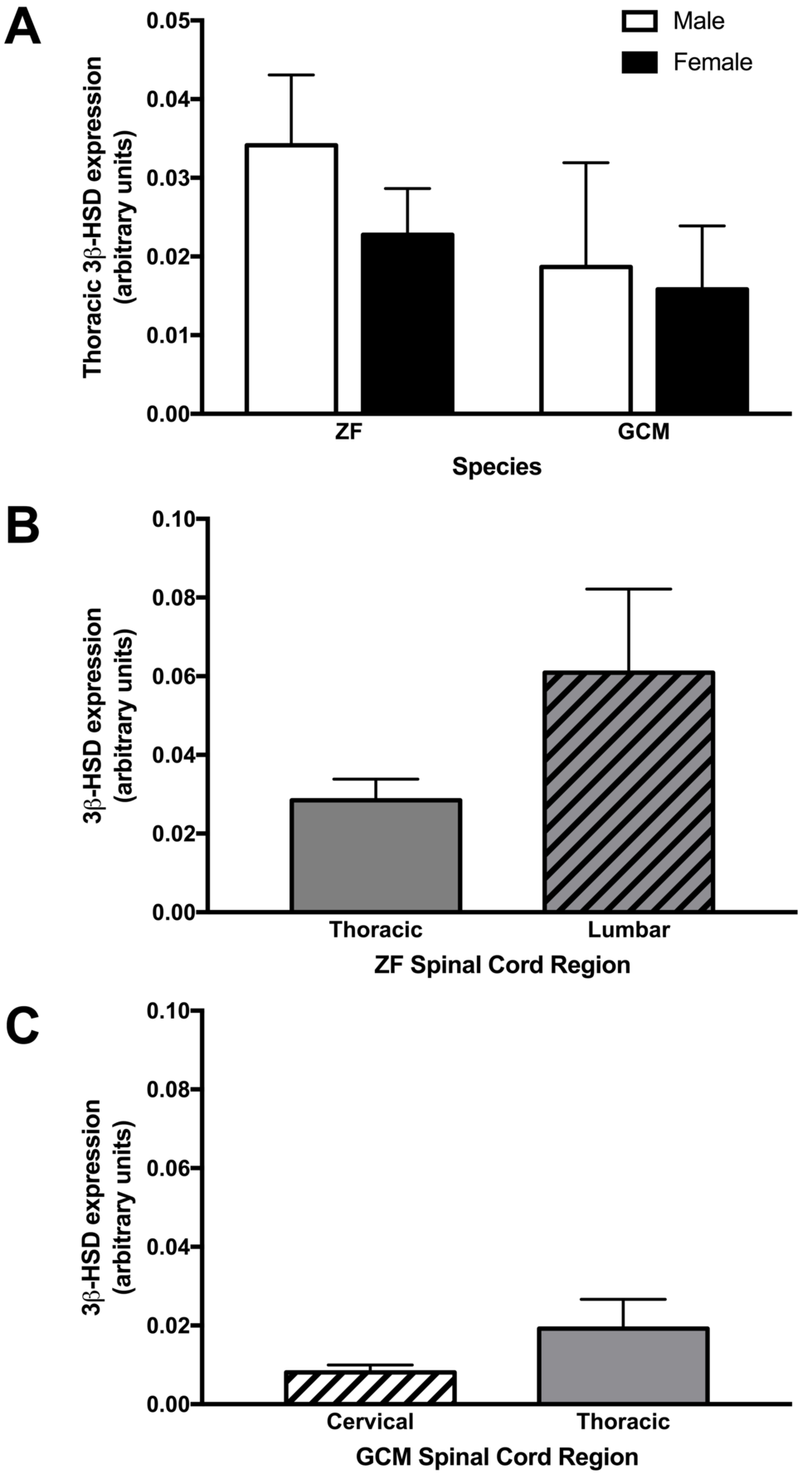
A
Male
Female
Thoracic 3β-HSD expression (arbitrary units)
0.05
0.04
0.03
0.02
0.01
0.00
ZF
GCM
Species
B
3β-HSD expression (arbitrary units)
0.10
0.08
0.06
0.04
0.02
0.00
Thoracic
Lumbar
ZF Spinal Cord Region
C
3β-HSD expression (arbitrary units)
0.10
0.08
0.06
0.04
0.02
0.00
Cervical
Thoracic
GCM Spinal Cord Region

Table 1
For each CNS region, the number of individuals of each species that had qPCR reactions of 3β-HSD mRNA expression with Ct values < 35.

Species	CNS Region	Male	Female	Total	% reaching criterion
	Brain				
Manakin	Cerebellum	1/6	2/6	3/12	25
	Hypothalamus	5/6	6/6	11/12	92
	Telencephalon	0/6	1/6	1/12	8.3
Zebra Finch	Cerebellum	5/5	6/6	11/11	100
	Hypothalamus	4/6	5/6	9/12	75
	Telencephalon	4/6	5/6	9/12	75
	Spinal Cord				
Manakin	Cervical	2/4	3/5	5/9	55.6
	Thoracic	2/4	3/5	5/9	55.6
	Lumbar	1/4	0/5	1/9	11.1
Zebra Finch	Cervical	0/5	1/5	1/10	10
	Thoracic	3/5	5/5	8/10	80
	Lumbar	4/5	4/5	8/10	80