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

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

A thesis submitted in partial satisfaction of the requirements for the degree
Master of Science in Physiological Science

by

Joy Eaton

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ABSTRACT OF THE THESIS

3β-HSD expression in the CNS of a manakin and finch

by

Joy Eaton

Master of Science in Physiological Science

University of California, Los Angeles 2015

Professor Barnett Schlinger, Chair

DHEA (dehydroepiandrosterone) can circulate at relatively high levels with a variety of reported actions on the central nervous system (CNS). Some of these actions require the conversion of DHEA into more active steroidal metabolites catalyzed by the enzyme 3β-HSD. Whereas DHEA is often measured in blood, efforts to evaluate neural 3β-HSD are limited. A role for neural 3β-HSD has been demonstrated in some non-breeding oscine songbirds when DHEA promotes aggressive behavior, most likely after its neural conversion into active androgens and estrogens. Our lab studied courtship behavior of male Golden-Collared Manakins (*Manacus vitellinus)* and found that low levels of courtship persist when circulating testosterone levels are basal. Therefore, we tested the hypothesis that DHEA might activate behavior in these sub-oscine birds by determining if DHEA is measurable in blood and by evaluating 3β-HSD expression in the brain and spinal cord. For comparison, we examined 3β-HSD expression in similar CNS tissues of zebra finches, an oscine species in which plasma DHEA and neural 3β-HSD expression have been reported previously. DHEA was detected in manakin blood at levels similar to that seen in other species. Although 3β-HSD was present in all finch

brain regions examined, 3β-HSD was expressed only in the manakin hypothalamus where it was present at relatively high levels. In the spinal cord, 3β-HSD was detected in some but not all regions examined in both species. These data indicate that manakins have the neural machinery to convert circulating DHEA into potentially active androgens and/or estrogens.

The thesis of Joy Eaton is approved.

David Walker

Paul Micevych

Barnett Schlinger, Committee Chair

University of California, Los Angeles

2015

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Introduction:

During periods of reproductive activity, gonadal testosterone (T) activates male aggressive, copulatory and courtship behavior(Wingfield, 1994; Wingfield and Monk, 1994; Goodson, 1998b; Soma et al., 1999c; Soma et al., 1999b; Soma et al., 1999a; Soma et al., 2000; Soma et al., 2002; Soma et al., 2003). In some bird species, steroid-dependent territorial aggressive behaviors are expressed outside of the breeding season when circulating T levels are low (Wingfield et al., 2001; Soma et al., 2002). At these times, the relatively inactive steroid dehydroepiandrosterone (DHEA), presumably secreted by the adrenals, can serve as the substrate for the formation in brain of active sex-steroids that stimulate territorial behavior (London et al., 2003; London et al., 2006; Schlinger and London, 2006; London et al., 2009; London et al., 2010). Upon interaction with the enzyme 3β-hydroxysteroid dehydrogenase/C17-20 lyase (3β-HSD), DHEA is converted to androstenedione which, in turn, can serve as the substrate for the formation of the more potent androgen testosterone (by action of the enzymes 17β-HSD) and also downstream products including 5α-DHT and 17β-estradiol, by action of the enzymes 5α-reductase and aromatase respectively (Schlinger and Arnold, 1991a; London et al., 2003; Soma et al., 2004; London et al., 2006; Fusani et al., 2014). Although DHEA may have some of its own neural actions (Majewska et al., 1990; Monnet et al., 1995; Goodson, 1998a; Park-Chung et al., 1999; Sperry et al., 2003; Robichaud and Debonnel, 2004), in all likelihood, these enzyme-catalyzed reactions are crucial for locally activating the relatively inert DHEA.

In birds, studies of neural 3β-HSD have focused largely on the brain, and examined Japanese quail and on species of oscine songbirds. (Usui et al., 1995; Tsutsui et al., 2003b, a; Tsutsui, 2008, 2011). Birds of the Order Passeriformes are separated into two following clades: the oscine songbirds, that learn complex songs from their father or tutor and possess a complex

neural circuitry that underlies song learning and expression and the sub-oscines, species that lack complex song and the most or all structures comprising the well-described oscine song system(Wade et al., 1995; Gahr and Wild, 1997; Holloway and Clayton, 2001; DeWulf and Bottjer, 2002; Grisham et al., 2002). Our lab has focused studies on the neuroendocrine basis of behavior in a sub-oscine species, the Golden-collared manakin (*Manacus vitellinus*) of Panamanian rainforests. Males of this species perform physically elaborate courtship displays daily, over the course of 6-7 month-long reproductive seasons. We have considerable evidence that these displays depend on androgens (Schlinger et al., 2008; Feng et al., 2010; Barske et al., 2011; Barske et al., 2014). Nevertheless, during the breeding season, circulating T levels in males are extremely variable with some displaying males having little or no measurable T levels in blood(Day et al., 2007; Fusani et al., 2007). Moreover, juvenile males during the breeding season and all males during the non-breeding seasons, birds with low levels of T, engage in low levels of courtship. We were curious if manakins had measurable DHEA in blood as well as neural 3β-HSD to catalyze the formation of more active sex-steroids in the brain.

In this current study, we measured DHEA in blood of adult breeding male golden-collared manakins and compared those levels to adult females and to juvenile non-breeding males. Because androgen and estrogen receptors are expressed in both the brain and spinal cord where steroids likely increase the motivation to perform male courtship and to coordinate motor performance (Schultz et al., 2001; Fuxjager et al., 2012a; Fuxjager et al., 2012b; Fuxjager et al., 2013; Schlinger et al., 2013) we used quantitative PCR to measure 3β-HSD mRNA expression in brain and spinal cord of adult males and female manakins. To ascertain if there might be differences between the sub-oscine manakin and an oscine songbird, we included adult male and female zebra finches in the expression analysis.

Experimental Procedures & Methods:

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 (n=12) samples were collected during the courtship season (February-April) from forests in and around Gamboa, Panama. Reproductively active zebra finches (n=12) were obtained from our UCLA colony.

Tissue Collection:

Blood samples were collected in Panama from 14 adult and 5 juvenile males and from 6 adult females. Animals were captured using mistnets and bled by venipuncture within 10 minutes of capture. Blood was kept on wet-ice or was refrigerated then centrifuged at 1000g within 3 hours to yield ~65 ul (30–100 ul) plasma. Manakin brain tissues were collected immediately upon decapitation, placed on dry ice and then stored either on dry ice or in a -80C freezer. Also, brains were dissected bilaterally (selected regions: cerebellum, hypothalamus, and whole telencephalon) and frozen immediately on dry-ice where they remained until placed in a -80C freezer at the Smithsonian Tropical Research Institute facilities in Panama City until shipped to UCLA; spinal cords were dissected into the cervical, thoracic and lumbo-sacral sections and flash frozen (both species at UCLA). These tissues were selected based on previous studies showing significant androgen and/or estrogen receptor expression in manakins suggesting their

possible function in activating and controlling male manakin courtship displays. (Shen et al., 1995; Cam and Schlinger, 1998; London et al., 2003; Soma et al., 2004; London et al., 2006; Feng et al., 2010)

DHEA measures:

Steroids were extracted from plasma using dichloromethane (DCM): plasma-volume varies, purified H2O-100ul and DCM-3ml. After centrifugation, samples were freeze-decanted and washed again with DCM. Samples were dried in a water bath and mixed with a diluent of 0.1% PBS and BSA .DHEA was quantified using a sensitive and specific radioimmunoassay as in Newman and Soma, 2011 (DSL8900, Beckman Coulter). 100 ul of suspended samples were used for the assay. High and low solutions the manufacturer provided controls. Complete protocol attached in supplemented material.

3BHSD Primers:

We utilized species-specific primers with quantitative PCR to examine 3β-HSD expression across sex, species and tissue. Primers made from PCR products ligated and sequenced by Genewiz.3βHSDprimer(F=AGGGCGTACTCGCTCGTCATCC/R=TAGAGCACGGTCAGAGGCATGG) was used to confirm that the specific adult Zebra Finch and Golden Collared Manakin primers are correct for the brain/ spinal cord tissues using PCR. Identity of sequence was confirmed by BLAST analysis (http://www.ncbi.nlm.nuh.gov/blast/) on the Zebra Finch genome.

RNA and PCR:

Total RNA was extracted from tissue samples using TRIzol Reagent ©(Invitrogen, Carlsbad, CA) and following the manufacturer's instructions. Tissues were homogenized for ~40 sec at medium/high speed with a standard stator homogenizer. RNA concentration was measured with a Nanodrop System 1000(Thermo Scientific, Wilmington, DE, USA), and its integrity was assessed using gel electrophoresis. 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. Resultant cDNA was used for PCR amplification to verify the presence of 3β-HSD transcripts in brain manakin and zebra finch brain and spinal cord. RT-PCR primers were designed from the zebra finch genome since there was high homologous representation between the avian species.

PCR reaction contained the following: 0.38 mM of deoxynucleotide triphosphate, 0.4 μM of forward and 0.4 μM of reverse primer, 50 ng of respective sample cDNA, 0.06 ng of DNA taq polymerase (Bioline, Randolph, MA) 2.5 KCL buffer, and 17.35 ul /2.9ul of sterile water for either primer or enzyme mix,. Reactions were run on Thermacycler at 95°C for 5 min and then subjected to 38 cycles of 95°C for 30 sec, ~64°C for 30 sec, 72°C for 1 min. Reactions were completed at 72°C for 10 min. PCR products for 3b-HSD were verified on gel electrophoresis to ensure that product size matched the expected base pair length. A smaple of the PCR amplification products were sequenced (Genewiz Inc., La Jolla, CA, USA) and blasted against the zebra finch genome confirming their identity as 3b-HSD.

Quantitative(RT)PCR:

To determine the relative abundance of 3b-HSD in manakin and zebra finch tissues we performed quantitativePCR (qPCR) using an ABI 7300-96 well sequence detection system with

SYBR Green PCR master Mix (Applied Biosystems Inc., Foster City, CA). All qPCR Primers (gene respective) were created from annotated Zebra Finch sequence; ZF primers were used on both species, since sequence data showed high homology across species. The housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was based on annotated zebra finch sequence and used for among all samples (F= TGACCTGCCGTCTGGAAAA; R= CCATCAGCAGCAGCCTTCA). GAPDH is a frequently generally since expression is unaffected by steroid treatment, and it's properties does not detectibly differ in primer binding and reaction efficiencies among this studies species . All qPCR reactions carried out as follows: 50°C for 2 min, 95°C for 10 min, and 40 cycles at 95°C for 15 sec/60°C for 1 min, with assay completed with 95°C for 15 sec, 60°C for 30sec, and 95°C for 15 sec (with a dissociation stage at the end of each reaction). Dissociation curves of the qPCR products were assed to assure absence of DNA contamination. All samples were ran in duplicate for both genes. Standard curves were determined beforehand, along with correlated coefficients and known concentration of cDNA, to generate slopes that could calculate amplification efficiency (90-110%) for each primer reaction.

Statistics:

DHEA levels were analyzed across adult males, adult females and juvenile males using One-way ANOVA. Delta Ct values were computed for each sample and relative abundance of 3β-HSD mRNA were analyzed separately for brains and spinal cords. For many individual samples, CT values were quite low. Therefore, we established a cut-off such that a CT of 35 or below was considered unreliable (Table 1). For each species, any tissue in which >50% of the samples were

at 35 or below were considered undetectable and were not analyzed further. For those samples subjected to statistical analysis, all CT values were included in the determination of deltaCT, with "undetermined" values assigned a CT value of 40. Delta Ct values were analyzed by two-way ANOVA with SPSS 22 program (sex* region/region * species) with significance accepted at $\alpha < 0.05$. This was not the case with the household gene which further proves that in some areas of the CNS 3β-HSD is not highly expressed. Furthermore, stats were done to ensure that the household gene was not creating the significant effects of expression. Delta Ct values were analyzed by two-way ANOVA with SPSS 22 program (sex* region/region * species) with significance accepted at $\alpha < 0.05$.

Results:

1. Plasma DHEA expression:

DHEA was detected in plasma of all 25 individuals examined with most falling between 1-2 ng/ml plasma . One-way ANOVA ($F(2,23)=1.187$;$P=.325$) found no significant differences across groups, though mean levels were slightly higher in adult females than in males(Figure 1).

2. 3β-HSD in manakin CNS:

We confirmed that manakins (as well as zebra finches) expressed 3β-HSD in selected CNS tissues. Using reverse-transcriptase PCR (rt-PCR), we amplified a single band from both species, that, upon sequencing, was a fragment of the 3β-HSD transcript (ESTIMA:http://titan

.biotec.uiuc.edu). The manakin PCR product was highly homologous (96.3%) to the zebra finch (www.genewiz.com).

3. Brain Regions:

Whereas 3β-HSD expression was detected in all brain regions of most zebra finches, reliable detection levels were only found in the hypothalamus of the manakin (Table 1; Figure 2). Therefore, we restricted our subsequent analysis to just the hypothalamus. Manakins had significantly higher hypothalamic 3β-HSD than did zebra finches ($F_{1,21} = 24.030$; $P=0.000$) with no sex ($F=1.284$; $P=0.271$) or sex* species interactions ($F=0.706$; $P=0.411$).

4. Spinal Cord Regions:

Reliable expression levels of 3β-HSD were found in cervical and thoracic regions of the manakins and in thoracic and lumbosacral regions of the zebra finch (Table 1; Figure 3). Therefore, we restricted our subsequent analysis across species to just the thoracic region where we found no significant species, sex or species*sex interactions ($F=2.786$; $P=0.362$; $F=1.036$; $P=2.791$; $P=0.469$). When species were analyzed separately, we found no significant region, sex or region* sex interactions for either species (Manakin: $F=0.191$; $P=0.669$; $F=0.761$; $P=0.394$; $F=1.187$; $P=0.325$/ZF: $F=1.302$; $P=0.267$; $F=2.680$; $P=0.986$; $F=1.574$; $P=0.598$).

Discussion:

Results presented here expand our appreciation of the potential pro-hormonal role of DHEA in birds by showing that a) DHEA is found to circulate in blood of a wild sub-oscine species, the golden collared manakin, at levels similar to that measured in other avian species; b) that the enzyme 3β-HSD is present at appreciable levels in the manakin hypothalamus and c) that 3β-HSD is expressed in the spinal cords of both Passeriformes species studied. Species differences in CNS 3β-HSD-expression point to possible functional differences across species that we discuss below.

Plasma DHEA:

In many mammals and birds, DHEA is found circulating at relatively high levels in both males and females after its synthesis largely in the adrenals glands. Moreover, DHEA often circulates at high levels in young adults and then declines with age (Soma et al., 1999b; Wingfield et al., 2001; Pradhan et al., 2010). Although no specific receptor for circulating DHEA has been identified, this hormone nonetheless has a variety of noteworthy effects on a variety of tissues, including in the CNS of humans and other animals(Cam and Schlinger, 1998; Holloway and Clayton, 2001; Soma et al., 2002; Soma et al., 2004; Schlinger et al., 2008).

In some species of songbirds, there is evidence that circulating DHEA in non-breeding males may activate sex steroid dependent aggressive behaviors (Soma et al., 2004; London et al., 2006; Tam and Schlinger, 2007; Schlinger et al., 2008; Pradhan et al., 2010). In all likelihood, this occurs after DHEA is converted into more active sex steroids locally in the brain by the enzyme 3β-HSD into androstenedione that is subsequently converted into active androgens and estrogens such as testosterone and 17β-estradiol (Schlinger et al., 1995; Cam and Schlinger,

1998; Day et al., 2006; Day et al., 2007; Day et al., 2011). In the absence of appreciable 3β-HSD elsewhere in the body, DHEA in blood can activate steroid-dependent circuits in the brain with little effect on other reproductive tissues.

We found that DHEA is readily detected in the blood of adult male and female manakins, as well as in blood of juvenile males. These levels are similar to what has been reported for other bird species. We detected no significant sex difference, though mean levels were somewhat higher in females. These data support the idea that DHEA is an important circulating hormone in these birds and adds sub-oscine species to the growing list of birds in which circulating DHEA can be detected. The absence of adult sex differences or age differences in males (as well as CNS 3β-HSD expression described below) argues that DHEA may not be involved in activating masculine courtship behavior at times when testosterone circulates at low levels. Thus, we assume that DHEA has alternate functions in these birds, such as follows: 1) DHEA can with rapid enzymatic conversation with 3β -HSD convert into active androgens /estrogens to modulate aggressive behaviors 2) Regulate sexual behaviors such as courtship displays in males 3) Undergo an indirect/ or direct hormonal effect through the HPG (Hypothalamic Pituitary Gonadal) Axis and 4) Protect and regulate the immune/nerve functions within this avian system. With such raised possibilities of DHEA having a dynamic contribution to the entire system, it will take more time to truly understand the mechanism of this specific molecule's vast reach. Ultimately, from this research and previous information, it would be correct to hypothesize that DHEA is necessary for homeostatic regulation overall in species.

3β-HSD expression:

3ß-HSD has been identified in the brains of several vertebrates with significant attention paid to the presence and function of this enzyme in the brains of birds ((Schlinger et al., 1995;

Wade et al., 1995; Vanson et al., 1996; Tam and Schlinger, 2007; London et al., 2009). Whereas this enzyme may participate in brain as part of a larger neurosteroidogenic cascade, whereby active steroids are synthesized de novo in brain from cholesterol (Schlinger and Callard, 1989a, b, 1990; Schlinger and Arnold, 1991b, a, 1992; Schlinger and London, 2006), there is also good evidence that 3β-HSD can function to catalyze the conversion of circulating DHEA locally in brain into more active sex steroid products (Soma et al., 1999c; Soma et al., 2002; Schlinger et al., 2008). For example, 3ß-HSD can cooperate with the estrogen synthetic enzyme aromatase in brain to convert DHEA into estradiol (London et al., 2003; London and Schlinger, 2007; London et al., 2009; London et al., 2010). Our results showing the 3β-HSD is expressed at relatively high levels in the manakin hypothalamus, where we have previously documented aromatase (Schlinger and Callard, 1989c, 1990; Schlinger and Arnold, 1991b, a, 1992; Vanson et al., 1996) lends support for the idea that manakin males, and perhaps also females, may synthesize estrogen in the hypothalamus from circulating DHEA substrate. As the hypothalamus contains neural circuits controlling reproductive behavior, DHEA might act indirectly within this region to influence the ways in which manakins perform their courtship behaviors. We cannot exclude the possibility that DHEA might also directly or indirectly, after conversion into androgenic or estrogenic metabolites, influence the manakin hypothalamic-pituitary-gonadal or -adrenal axes.

Interestingly, manakins express little if any 3β-HSD in the cerebellum and telencephalon, brain regions with reliably detectable levels in zebra finches (Delta Ct= 25.6789 avg). We had concluded that the relatively high expression of several steroidogenic enzymes in the oscine zebra finch brain, especially in the telencephalon, cerebellum and midbrain, might be associated with the complex neural circuitry they possess to control song and song learning. This current

data showing low expression of 3ß-HSD in the sub-oscine manakin telencephalon and cerebellum lends support for this hypothesis.

This latter conclusion differs from the alternate prediction that the complex courtship of manakins might require a significant action of steroids on diverse pre-motor circuits in the brain. For example, androgen receptors are expressed at relatively high levels in the manakin arcopallium (in the telencephalon), nucleus intercollicularis and in cerebellar Purkinje cells (Majewska et al., 1990; Monnet et al., 1995; Goodson, 1998b, a; Isgor and Sengelaub, 1998; Tsutsui, 2011) where DHEA could act, after conversion to more potent androgens, to activate male courtship. The current data do not support this mechanism.

Similarly, we might have expected to identify high levels of 3β-HSD in the spinal cord, especially in regions housing androgen-sensitive motor neurons that innervate skeletal muscles involved in the male manakin's complex courtship display. Although it is intriguing that 3β-HSD was expressed in regions of both the manakin and zebra finch spinal cords, the distribution of expression we observe lends little support for the idea that 3β-HSD is especially important in the manakin spinal cord or is related to complex courtship. It is important to appreciate that 3ß-HSD not only uses DHEA as a substrate, but can also convert pregnenolone into progesterone. Although we did not measure circulating 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 from local or circulating substrates, and that this progesterone exerts an influence on neural spinal circuits in both manakins as well as zebra finches.

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 both 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.

Table 1: 3β-HSD expression: CT values < 35

Species:	Brain	Male	Female	Total : % reaching criterion
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	11/12; 92%
	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-Sacral	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-Sacral	4/5	4/5	8/10; 80%

To measure relative abundance of 3β-HSD mRNA in male and female (Zf & Wild Manakin) brain/spinal cord regions, individual samples from each animal subject were subjected to rqRT-PCR. The Delta Ct values were analyzed via two-way ANOVA($\alpha<0.05$) with SPSS 22 program (sex* region/region * species). Also, a criterion was established to ensure proper expression of 3BHSD. The criteria is as followed:(illustrated in Table #1)

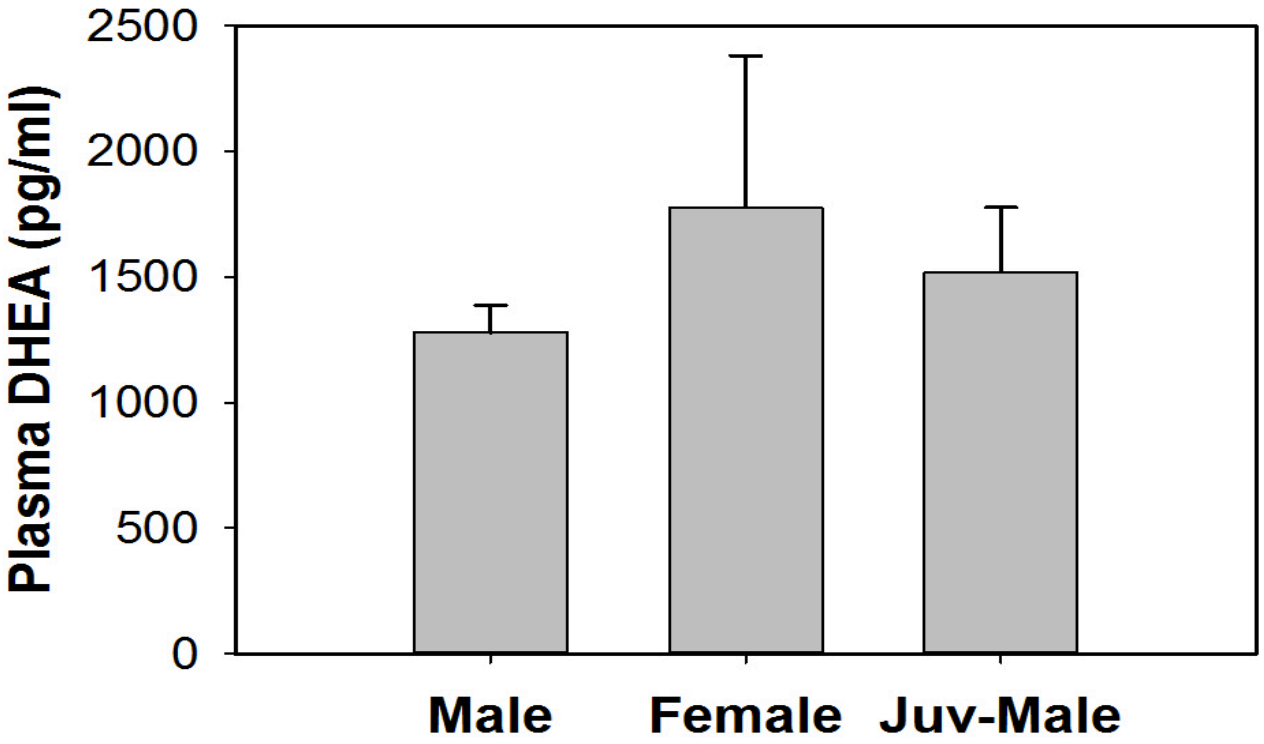
1) Individual sample's CT value must be below 35, then expression is considered present and sample utilized in ANOVAs analysis.

2) If individual sample's CT value is above 35 or undetermined, expression is considered absent and sample is removed before any SPSS analysis of data set.

3) If a region (regardless of species or sex) has a pass rate of 50% or greater, CT values of all samples(including those undetermined)will be included before SPSS analysis of data.

4) Undetermined values are given raw CT value of 40 before analyzed into a Delta CT value for regions with a pass criterion.

5) All analyzed stats by criterion must be parametric and tested for normality by using residuals.



Figure# 1: Plasma DHEA levels in male (n=14), female (n=6), and juvenile male (n=5) Golden collared manakins.

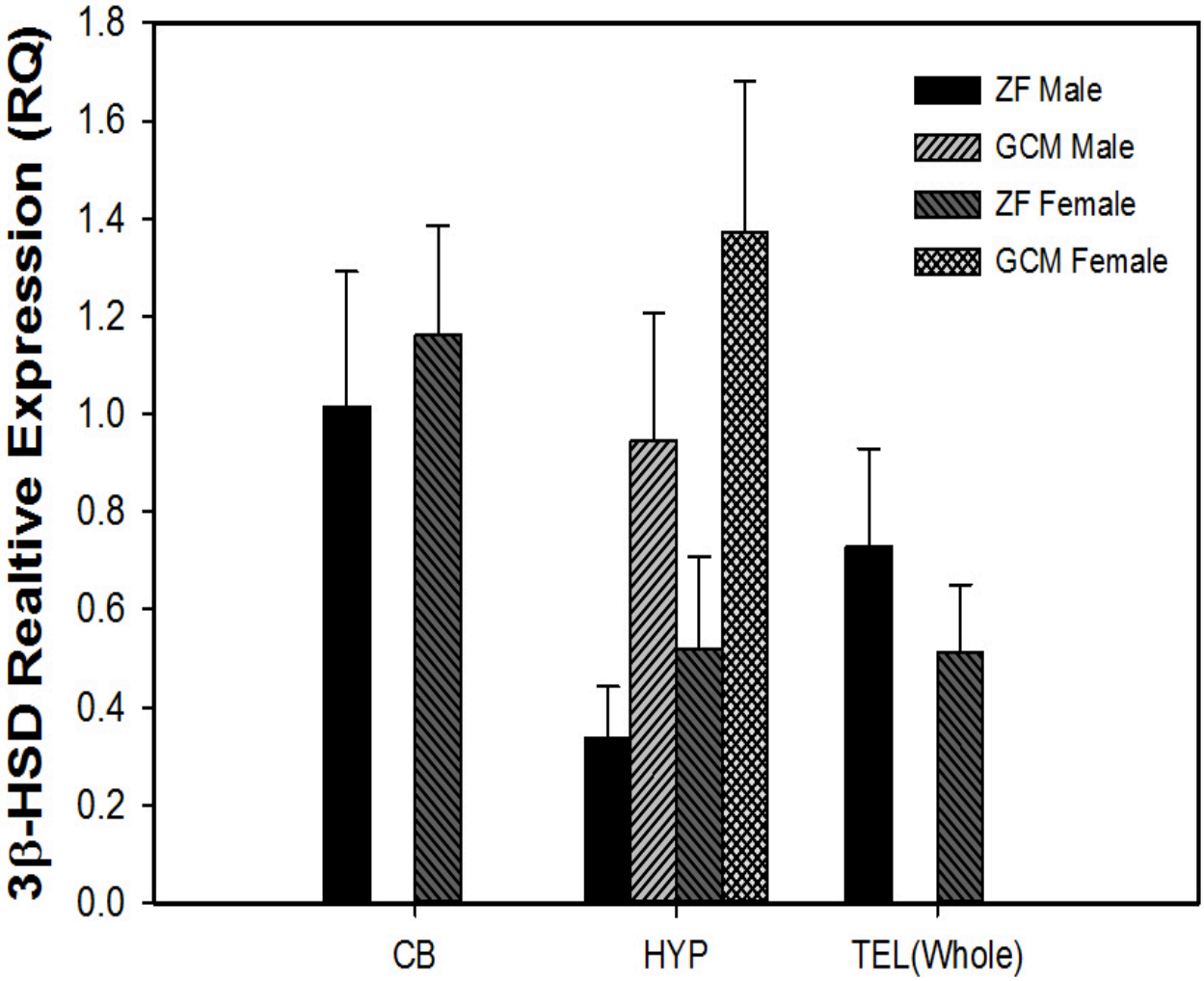


Figure 2: Relative 3β-HSD expression across three brain regions (Cb-cerebellum; Hyp-hypothalamus; Tel-telencephalon) for male and female golden collared manakins (Man) and zebra finches (ZF). Expression was undetected in manakin CB and Tel but expression in hypothalamus was significantly greater in manakin than in zebra finch.

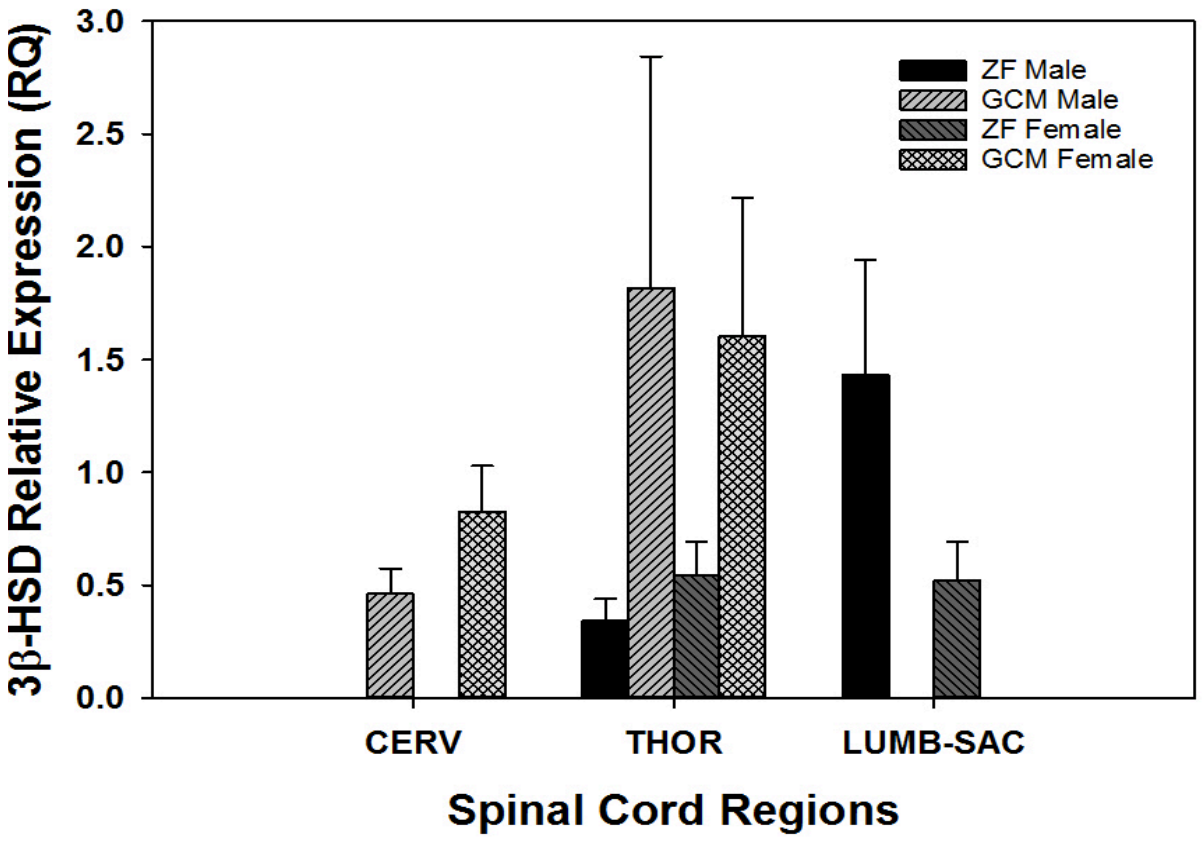


Figure 3: Relative 3β-HSD expression across three regions of the spinal cord (Cerv=cervical; Thor-thoracic; Lumb/Sac-lumbosacral) in male and female golden collared manakins (Man) and zebra finches (ZF). Expression was undetected in cervical region of the ZF and lumbosacral region of the manakin. There were no significant differences across sex, species or sex*species interaction for the thoracic region. There was no within species differences across region, sex or region*sex interaction for either species.

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