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Sex steroid profiles and pair-maintenance behavior of captive wild-caught zebra finches (*Taeniopygia guttata*)

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Abstract Here, we studied the life-long monogamous zebra finch, to examine the relationship between circulating sex steroid profiles and pair-maintenance behavior in pairs of wild-caught zebra finches (paired in the laboratory for >1 month). We used liquid chromatography–tandem mass spectrometry to examine a total of eight androgens and progestins [pregnenolone, progesterone, dehydroepiandrosterone (DHEA), androstenediol, pregnan-3,17-diol-20-one, androsterone, androstanediol, and testosterone]. In the plasma, only pregnenolone, progesterone, DHEA, and testosterone were above the limit of quantification. Sex steroid profiles were similar between males and females, with only circulating progesterone levels significantly different between the sexes (female > male). Circulating pregnenolone levels were high in both sexes, suggesting that pregnenolone might serve as a circulating prohormone for local steroid synthesis in zebra finches. Furthermore, circulating testosterone levels were extremely low in both sexes. Additionally, we found no correlations between circulating steroid levels and pair-maintenance behavior. Taken together, our data raise several interesting questions about the neuroendocrinology of zebra finches.

Keywords Affiliation · Pair bond · Songbird · Steroid profiling · Opportunistic breeder

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Introduction

Monogamy occurs across a wide range of species, including primates, rodents, birds, reptiles and fish (Reichard and Boesch 2003). Importantly, there is considerable variation within monogamous mating systems (Black 1996; Reichard and Boesch 2003). Pair bonds can be transient (lasting one breeding cycle) or life-long (Reichard and Boesch 2003). For species that form transient pair bonds, individuals repeatedly invest time and effort in courtship and pair-bond formation; however, it is unclear whether significant investment is needed to maintain these short-term bonds. In sharp contrast, for species that form life-long pair bonds, it is arguable that the ability to maintain a pair bond is equal to or more important than the ability to form the initial bond. While the importance of pair bonds has long been recognized in ethology (Silcox and Evans 1982; Beletsky 1983; Evans and Poole 1984; Black 1996), relatively little is known about the neuroendocrine regulation of pair-bond maintenance.

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The specific affiliative behaviors associated with courtship, pair-bond formation, and pair-bond maintenance are often very similar. However, the neuroendocrine regulation of an operationally defined behavior can differ across contexts (Hessler and Doupe 1999; Heimovics and Riters 2008; Oliveira 2009). Furthermore, the neuroendocrine mechanisms regulating the initial expression of a behavior often differ from those regulating the maintenance of that behavior (Carter 1998; Adkins-Regan 2005). Therefore, it is possible that neuroendocrine mechanisms regulating pair-bond maintenance are different from those regulating pair-bond formation. Indeed, the few studies that have directly examined the neuroendocrine regulation of pair-bond maintenance versus formation suggest that they are different (*prairie voles*: Aragona et al. 2006; Resendez and Aragona 2013; *zebra finches*: Smiley et al. 2012). The majority of research on the neuroendocrinology of pair bonding has focused on initial bond formation (Prior and Soma 2015).

Zebra finches (*Taeniopygia guttata*) form genetically monogamous life-long pair bonds (Zann 1996; Griffith et al. 2010). As an adaptation to the unpredictable environmental conditions in central Australia, zebra finches may have evolved long-term pair bonds to facilitate breeding flexibility and to allow pairs to rapidly initiate breeding bouts under favorable environmental conditions. Thus, there may be fitness benefits for pairs that form life-long bonds rather than transient bonds (Zann 1996; Adkins-Regan and Tomaszycki 2007). In fact, strong pair bonds are associated with greater reproductive success within a single breeding attempt (Mariette and Griffith 2012).

Beyond courtship behavior, there is mixed evidence implicating sex steroids in zebra finch pair bonding (reviewed in Prior and Soma 2015). Taken together, the evidence suggests that social and environmental contexts interact with sex steroids to modulate pairing behavior (Prior and Soma 2015). Using an open aviary behavioral assay, Tomaszycki et al. (2006) found no significant effects of administering an aromatase inhibitor (1,4,6-androstatriene-3,17-dione, ATD) and an androgen receptor antagonist (flutamide) on pair-bond formation. In contrast, using a partner separation and reunion test, we found that administration of an aromatase inhibitor (fadrozole) rapidly increases the amount of time spent in close proximity, in established zebra finch pairs (Prior et al. 2014). Furthermore, male and female zebra finches have high levels of sex steroids in behaviorally relevant brain regions (Prior et al. 2013). It is also likely that sex steroids modulate other signaling molecules, which are also implicated in the regulation of pairing behavior (Banerjee et al. 2013; Klatt and Goodson 2013; Prior and Soma 2015).

Liquid chromatography–tandem mass spectrometry (LC–MS/MS) can measure multiple steroids simultaneously with higher specificity than immunoassays (Koren et al. 2012). Measuring multiple steroids has several benefits. First, circulating levels of sex steroid precursors (e.g., dehydroepiandrosterone, DHEA) rather than the sex steroids themselves can be correlated with social behaviors, such as aggression (Soma et al. 2004, 2008; Fokidis et al. 2013). Second, characterizing sex steroid profiles elucidates "upstream" and "downstream" endocrine mechanisms. For example, active sex steroids can be produced locally in the brain from inactive precursors (prohormones) in the blood, and thus it is valuable to measure circulating levels of prohormones (Schmidt et al. 2008). Third, LC–MS/MS can measure less commonly studied steroids, such as those in

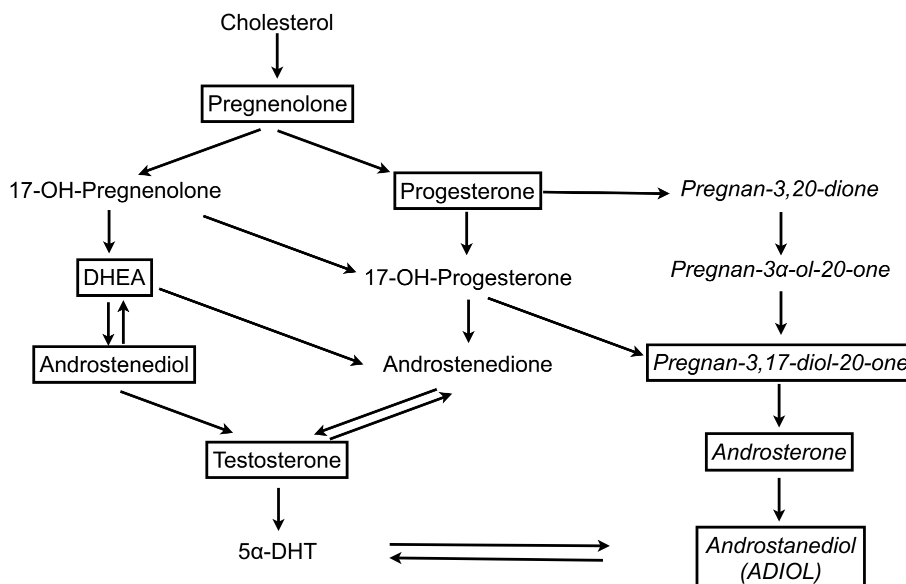

Fig. 1 Steroidogenic pathway highlighting the traditional pathways to 5α-DHT and the "backdoor" pathway to 5α-DHT (*italicized*). Steroids that we examined with LC–MS/MS are *boxed*. In the plasma, four steroids were present at levels above our limit of quantification (LOQ): pregnenolone, progesterone, DHEA, and testosterone. Trace levels [above the limit of detection (LOD) but not the LOQ] were present for androstenediol and androsterone

the "backdoor" pathway to 5α-dihydrotestosterone (5α-DHT) (Fig. 1) (Auchus 2004). The backdoor pathway was identified in some mammalian species, but has been nearly unstudied in birds (Fokidis et al. 2015). The backdoor pathway to 5α-DHT by-passes testosterone (Fig. 1) and might be important for local steroidogenesis when systemic levels of testosterone are low (Auchus 2004).

Here, we used LC–MS/MS to measure sex steroid profiles of wild-caught zebra finches that were paired in captivity. We examined eight androgens and progestins: pregnenolone, progesterone, DHEA, androstenediol, pregnan-3,17-diol-20-one, androsterone, androstanediol (ADIOL), and testosterone. This allowed us to (1) identify which steroids are present in the circulation of wild-caught zebra finches and (2) examine relationships between steroid profiles and pair-maintenance behavior. Based on our previous results, we predicted that circulating androgens would be positively correlated with pair maintenance (Prior and Soma 2015). Additionally, previous work in developing rodents and tammar wallabies (Shaw et al. 2000) suggests that, if the backdoor pathway is used, ADIOL would be elevated in the circulation.

Materials and methods

Subjects

Wild zebra finches ($N = 22$ individuals) were caught on Fowlers Gap Arid Zone Research Station, NSW, Australia (Griffith et al. 2008) in September 2012 (austral spring), during a period when breeding was occurring in adjacent areas of the field station, and placed into outdoor aviaries (220 cm × 160 cm × 200 cm). All aviaries had natural lighting and a one-way viewing screen for behavioral observations. Subjects were given access to seed and water ad libitum. Subjects ($N = 11$ males and 11 females) were placed in five aviaries that housed four birds each (2 males and 2 females) and in a sixth aviary that housed two birds (1 male and 1 female). As the wild birds were initially unmarked, we were unable to ascertain whether any of the birds were paired with each other previously, and thus the birds are likely to have established new pairings at the start of this experiment. For this reason, the birds were left largely undisturbed for 1 month prior to data collection.

The aviaries were outfitted with nest boxes and nesting materials (natural grasses from the area), and we continued to supply nesting materials throughout the study. All of the newly established pairs engaged in nest building. However, during the 2½ months of this study, only one pair bred. Most of these pairs began breeding following the completion of this study and there was an abundance of breeding throughout the nestbox colonies of Fowlers Gap, and thus these captive pairs were in a "pre-breeding" state for this study.

Identification of pairs

All birds were banded with a colored leg band for identification. At the end of the 1-month habituation period, one researcher (NHP) observed the individuals to identify pairs. Even when not breeding, pairs build and maintain a roost nest (Kikkawa 1980; Zann 1996). Paired individuals selectively engage in affiliative behavior with their partner (e.g., allopreening and clumping) (Zann 1996; Elie et al. 2011). Thus, pairs were defined as male–female dyads found clumping together (either inside or outside a nest). Pairs were stable for the duration of the study.

General timeline

Behavioral observations and blood samples were collected at three timepoints: timepoint 1 (day 0, this occurred after the 1-month habituation period), timepoint 2 (~day 31), and timepoint 3 (~day 38). For the one pair that bred, we observed behavior and collected blood samples at times that corresponded to key points during the breeding cycle. For this one pair, timepoint 2 corresponded to incubation (day 19) and timepoint 3 corresponded to chick-rearing (day 34).

Behavioral observations

At each timepoint we conducted four, 10-min behavioral observations, two morning (0800–1200 h) and two evening (1700–1900 h) observations. Typically morning and evening observations were collected from consecutive days. At timepoint 1, eight of the pairs had one evening observation. Thus we have a total of 11 or 12 behavioral observations for every pair. At each behavioral observation, one observer (NHP) stood outside the aviary and scored behavior through the one-way viewing screen. Behavioral scoring was spoken into a microphone and later transcribed.

We scored several types of behavior, including foraging, affiliative behavior, and aggression. Five behaviors were classified as affiliative: clumping (in physical contact and facing the same direction while perched), allopreening, coordinated preening (male and female self-preening at the same time), co-nesting, and overall time engaged in coordinated activities (coordinated foraging, preening, and nesting). Occasionally, we did observe chases associated with nest defense, however those behaviors were extremely rare (data not shown). The most common affiliative behaviors were clumping and co-nesting, and thus we used these to determine total time spent "affiliating" (see "Results").

Brachial blood samples

For each timepoint, blood samples were collected from the brachial vein of both the male and female of each pair (between 0800 and 1300 h). All individuals in an aviary were caught and bled within 13 min (mean ± SEM 6.9 ± 0.40 min) of entering the aviary. All subjects in an aviary were sampled at the same time to reduce the effect of stress from repeated chases.

Generally, the blood samples were collected 1–2 days after the last behavioral observation (1.5 ± 0.1 days). For timepoint 1, blood samples were collected 2 days before the first behavioral observations for seven pairs and 1–2 weeks after the first behavioral observations for four pairs. Plasma was obtained by centrifugation of heparinized capillary tubes. Samples were kept frozen until further processing. Of the 66 samples, 62 were successfully analyzed (2–3 plasma samples per subject).

Steroid analysis: liquid chromatography–tandem mass spectrometry

Based on pilot work, steroid extraction and LC–MS/MS protocols were adapted for avian plasma from the protocol in Adomat et al. (2012). These changes included a longer LC gradient, multiple reaction monitorings for additional steroids, and modified extraction conditions. A brief summary of our finalized protocol is described below. High extraction efficiencies and low ion suppression allowed the use of neat steroid standards for calibration, similar to Adomat et al. (2012). Extracts from equivalently spiked charcoal-stripped serum showed negligible accuracy bias.

Samples (~30 μL of plasma) were adjusted for pH using 20 μL of 1 M NaOH, and then deuterated testosterone, 5α-DHT and ADIOL (d3-testosterone, d3-DHT, d3-ADIOL, C/D/N Isotopes Inc., QC, Canada) were added as internal standards. Steroids were then extracted using 2,000 μL of 60:40 (v/v) hexane:ethyl acetate for 30 min, and the upper layer was collected. This was followed by two similar extractions, which were pooled with the initial extract. This modified extraction protocol ensured a high extraction efficiency. Pooled extracts were dried in a centrifugal vacuum evaporator (Centrivap, Labconco).

The resulting residues were derivatized using 2-fluoro-1-methylpyridinium *p*-toluene-4-sulfonate (FMP, Sigma, Oakville, ON, Canada), which enhances sensitivity for hydroxylated steroids. More specifically, FMP was dissolved in dichloromethane to yield a 20 mM solution, and then 4 μL/mL of triethylamine was added. This solution was prepared immediately prior to use. Individual samples were then treated with 400 μL of the FMP solution and allowed to react at room temperature for 1 h. Then 50 μL of methanol was added to quench any residual reagent for 15 min, after which the samples were again dried in the Centrivap. The dried extracts were then reconstituted in 50 μL of 50 % methanol, centrifuged at 20,000 *g* for 5 min to sediment any remaining particulates, and transferred into LC vials with low volume inserts for analysis by LC–MS/MS.

Analysis was carried out with a Waters Acquity UPLC Separations Module coupled to a Waters Quattro Premier XE Tandem Mass Spectrometer (Waters Corporation, MA, USA). A 2.1 × 100 mm BEH 1.7 μM C18 column was used for the steroid samples (Waters Corporation, MA, USA). The mobile phases were water and acetonitrile, both containing 0.1 % formic acid, using the following gradient: 0 min, 10 %; 0.5 min, 10 %; 1 min, 20 %; 7 min, 30 %; 13 min, 35 % (%acetonitrile). This was followed by a column flush of 95 % acetonitrile and re-equilibration, for a total run length of 18 min. Column temperature was 35 °C, and injection volume was 15 µl. The mass spectrometer was set at unit resolution, capillary was 3 kV, source and desolvation temperatures were 120 and 300 °C, respectively, desolvation and cone gas flows were 1,000 and 50 L/h, and the collision cell pressure was held at 4.6×10^{-3} mbar. All data were collected in electrospray (positive mode) (ES+) by multiple reaction monitoring for steroids. Instrument parameters were optimized for the mass to charge ratio (*m*/*z*) values, and corresponding fragments of the oxime-steroids monitored for each multiple reaction monitoring.

Steroid quantification

Data processing was conducted using Quanlynx (Waters Corporation, MA, USA) and exported to Excel for further analysis. Peak area-under-the-curve ratios were used for quantification, with d3-DHT as the internal standard for all steroids but T and ADIOL.

Calibration samples consisted of neat standards (6 standards ranging from 0.02 to 10 ng/mL), and observed steroid levels were normalized to sample volume. The limit of detection (LOD) ranged from approximately 0.01 to 0.02 ng/mL (3× the background); the limit of quantification (LOQ) was approximately 10× background (±20 % accuracy). Recoveries and conversions to derivatized steroid species were >80 % for each steroid.

Statistics

All statistics were conducted in R v 2.12.2 (R Core Team 2012). Figures were made in R and GraphPad Prism (GraphPad Software Inc., CA, USA).

Given that only one pair changed their breeding status during the experiment and that initial statistical models showed no effect of Timepoint (within-subjects factor,

rmANOVA), sex steroid levels were averaged across the three Timepoints for each individual (see "Results"). To determine if there was a sex difference in steroid levels, Mann–Whitney tests were conducted. Additionally, linear regressions were conducted to determine if there was a relationship between (1) female and male steroid levels within a pair, and (2) levels of different steroids within an individual. Data were transformed as necessary.

Time spent affiliating was not affected by Timepoint (within-subjects factor, rmANOVA), and thus time spent affiliating was averaged across the three timepoints for each pair. To determine if sex steroid profiles correlated with pair-maintenance behavior, we conducted linear regressions on the female and male sex steroid levels and time spent affiliating (%). Data were transformed as necessary.

Results

Sex steroid profiles

Of the eight steroids that we investigated, two were below our LOD in every plasma sample: pregnan-3,17-diol-20-one and androstanediol (ADIOL). We were able to detect trace amounts (>LOD) of androstenediol and androsterone in a small number of samples (Table 1). Importantly, the 5α-reduced steroid precursors associated with the backdoor pathway were largely non-detectable in plasma, despite the low detection limit of the LC–MS/MS assay. Specifically, circulating ADIOL was non-detectable, which we had predicted would be elevated if the backdoor pathway was being used.

Four steroids were present in plasma at concentrations above our LOQ: pregnenolone, progesterone, DHEA, and testosterone (Table 1). Circulating steroid levels did not significantly differ across the three timepoints (pregnenolone: $F_{(1,58)} = 1.04$, $P = 0.312$; progesterone: $F_{(1,58)} = 0.24$, $P = 0.625$; DHEA: $F_{(1,58)} = 0.01$, $P = 0.917$; testosterone $F_{(1,58)} = 1.07$, $P = 0.306$). Thus, steroid levels were averaged across the three timepoints for each individual.

Circulating pregnenolone, DHEA and testosterone were not significantly different in females and males (Fig. 2a, c, d: pregnenolone: $W = 62$, $P = 0.949$; DHEA: $W = 77$, $P = 0.300$; testosterone: $W = 44$, $P = 0.277$). Circulating progesterone levels were higher in females than in males (Fig. 2b: progesterone: $W = 92$, $P = 0.041$). There was no relationship between female and male levels of any steroid within a pair (pregnenolone: $R^2 = 0.04$, $P = 0.568$; progesterone: $R^2 = 0.0005$, $P = 0.947$; DHEA: $R^2 = 0.13$, $P = 0.280$; testosterone: $R^2 = 0.05$, $P = 0.525$). In both females and males, circulating pregnenolone levels were correlated with circulating DHEA levels (females: $R^2 = 0.45$; $P = 0.023$; males: $R^2 = 0.89$; $P < 0.001$). In contrast, circulating pregnenolone levels were not correlated with circulating testosterone levels (females: $R^2 = 0.14$; $P = 0.250$; males: $R^2 = 0.01$; $P = 0.762$).Interestingly, circulating pregnenolone concentrations were an order of magnitude higher than any other steroid measured (Table 1). Additionally, circulating testosterone levels were extremely low (<0.2 ng/mL) (Table 1; Fig. 2d).

Behavior of pairs

A summary of common behaviors, including affiliation, nesting, preening and foraging behavior is presented in Table 2. Co-nesting and clumping were the most common affiliative behaviors (Table 2). Thus, time spent nesting (coordinated) and clumping were summed (time spent affiliating) within each behavioral observation. Overall, time spent affiliating was higher in the evening ($W = 36$, $P = 0.014$). Time spent affiliating in the morning and evening were correlated across pairs ($R^2 = 0.71$; $P = 0.001$);

Table 1 Descriptive statistics for circulating steroid levels in captive wild-caught zebra finches analyzed by LC–MS/MS

	Pregnenolone	Progesterone	DHEA	Androstenediol	Pregnan-3,17-diol-20-one	Androsterone	ADIOL	Testosterone
% Total samples > LOD	100	90	100	19	0	5	0	44
% Total samples > LOQ	100	48	100	0	0	0	0	31
Min (ng/mL)	0.23	0.04	0.03					0.04
Max (ng/mL)	18.88	1.81	1.67					1.60
Mean ± SEM (ng/mL)	5.54 ± 0.50	0.41 ± 0.09	0.46 ± 0.04					0.26 ± 0.08

Samples were collected from females ($N = 29$ samples from 11 females) and males ($N = 33$ samples from 11 males). The percent of samples greater than the limit of detection (LOD) and the subset of those greater than the limit of quantification (LOQ) are given. For the samples that were greater than the LOQ, descriptive statistics are given. For androstenediol and androsterone, some samples were above the LOD, but no samples were above the LOQ

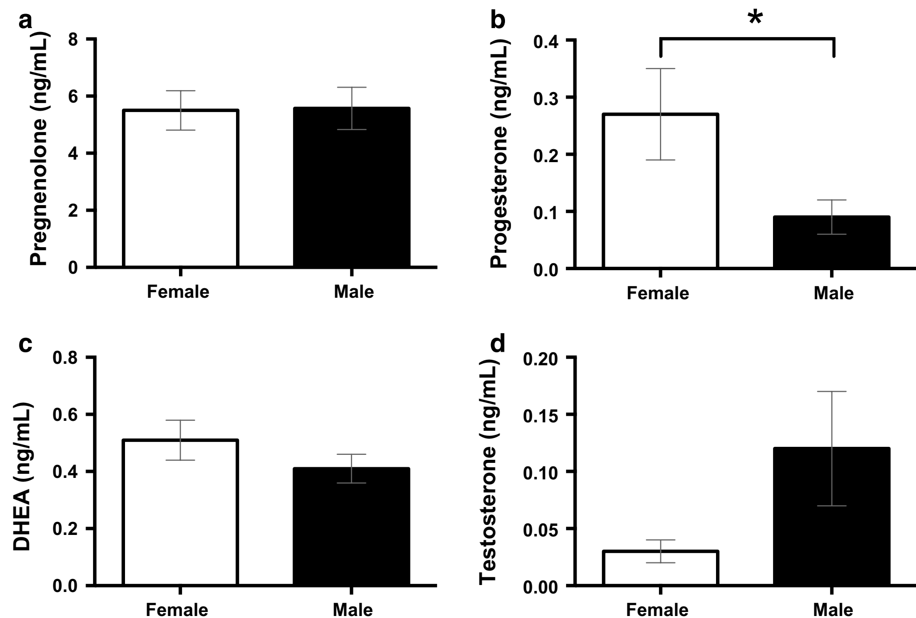

Fig. 2 Circulating levels of pregnenolone (**a**), DHEA (**c**), and testosterone (**d**) were not significantly different in males and females. Circulating progesterone levels (**b**) were higher in females than in males ($W = 92$, $P = 0.041$). Overall, circulating levels of these steroids were very low, with the exception of pregnenolone. Circulating pregnenolone levels were an order of magnitude greater than those of other steroids examined. *$P < 0.05$

Table 2 Behaviors in the morning and evening

	Morning	Evening
Affiliation		
Clumping	12 ± 5	51 ± 18
Allopreening	2 ± 1	6 ± 3
Nesting		
Female	35 ± 14	50 ± 19
Male	20 ± 8	36 ± 14
Coordinated	36 ± 15	83 ± 24
Preening		
Female	3 ± 1	10 ± 3
Male	16 ± 5	14 ± 3
Coordinated	4 ± 1	6 ± 3
Foraging		
Female	1 ± 1	6 ± 5
Male	2 ± 1	1 ± 1
Coordinated	1 ± 1	1 ± 1

Average time (s) spent engaging in behaviors per 10 min observation (in the morning and evening) (mean ± SEM)

thus for each pair we averaged time spent affiliating for the three to four observations per timepoint. Furthermore, time spent affiliating did not significantly differ across the three timepoints ($F_{(1,120)} = 1.29$, $P = 0.258$). Therefore, time spent affiliating was averaged across the three timepoints for each pair and compared to circulating sex steroid profiles within pairs. Despite variation in both time spent affiliating and circulating steroid levels of males and females, there was no relationship between steroid levels and pair-maintenance behavior (Table 3; Fig. 3).

Discussion

By using LC–MS/MS, we were able to develop a broader sex steroid profile for zebra finches and to correlate these sex steroid profiles with pair-maintenance behavior. While the pairs did vary in time spent affiliating, there was no relationship between this variation and sex steroid profiles of males and females. However, our results do raise several important questions regarding the neuroendocrinology of zebra finches. Sex steroid profiles were similar in males and females. In addition, both males and females had circulating pregnenolone levels an order of magnitude higher than the other steroids, highlighting the potential importance of this largely understudied prohormone. In contrast to high pregnenolone levels, other circulating steroid levels were relatively low. Importantly, despite a sensitive assay, we did not detect any circulating ADIOL, providing no evidence (at least from plasma) that the backdoor pathway is used in zebra finches. Taken together, these results raise the hypothesis that local steroid production may be elevated in wild zebra finches.

Sex steroid profiles

The circulating testosterone levels reported here are surprisingly low: ~0.12 ng/mL in males and ~0.03 ng/mL in females. Circulating testosterone has been measured in non-domesticated or wild zebra finches only two times previously (Vleck and Priedkalns 1985; Perfito et al. 2007). Using a radioimmunoassay (RIA), Perfito et al. (2007) reported slightly higher circulating testosterone levels

Table 3 R^2 values between circulating steroid levels and pair-level affiliative behavior

	R^2	*P* value
Pregnenolone		
Female	0.31	0.073
Male	0.04	0.567
Progesterone		
Female	0.31	0.078
Male	0.12	0.291
DHEA		
Female	0.002	0.887
Male	0.006	0.812
Testosterone		
Female	0.004	0.853
Male	0.18	0.183

(0.2–0.5 ng/mL in males and 0.14–0.15 ng/mL in females, depending on habitat), but found fewer detectable samples than the present study (36 vs. 44 %). Vleck and Priedkalns (1985) pooled plasma samples from 5 to 16 individuals, and testosterone levels were still close to the detection limit (<0.3 ng/mL) in males. Together, these studies and the present study suggest that circulating testosterone levels might be lower in non-domesticated than domesticated zebra finches. In domesticated zebra finches, plasma testosterone levels in males are often 1–3 ng/mL (Adkins-Regan et al. 1990; Kabelik et al. 2011; Prior et al. 2013). However, it is possible that captivity (rather than domestication per se) influences the HPG axis (Calisi and Bentley 2009; Dickens and Bentley 2014).

In addition, the methods used to quantify steroids differ across studies. LC–MS/MS is highly specific, and we expected our values to be slightly lower than those determined with immunoassays. Circulating testosterone levels in domesticated zebra finches have been quantified with LC–MS/MS and are higher than the testosterone levels we report here (Koren et al. 2012; Prior et al., unpublished results). To confirm that our results were similar using a RIA, we used a validated RIA protocol (Prior et al. 2013) to quantify testosterone in a subset of eight plasma samples from zebra finches in this study, and we saw similarly low levels of circulating testosterone (mean ± SEM ng/mL: male, $N = 4$, 0.14 ± 0.04; female, $N = 4$, 0.08 ± 0.01).

To our knowledge, none of the other steroids examined here have been quantified in non-domesticated zebra finches. Circulating DHEA and progesterone levels reported here are slightly lower than, but similar, to levels reported in domesticated zebra finches using LC–MS/MS (Koren et al. 2012) and are lower than levels reported in domesticated finches using RIA (progesterone: Pröve1983; Taves et al. 2016; DHEA: Soma et al. 2004; Fokidis et al. 2013; Prior et al. 2013). Importantly, DHEA and progesterone may regulate social behavior in zebra finches (progesterone: Smiley et al. 2012; DHEA: Fokidis et al. 2013). Furthermore, DHEA can be rapidly metabolized into active steroids in the zebra finch brain (Pradhan et al. 2008).

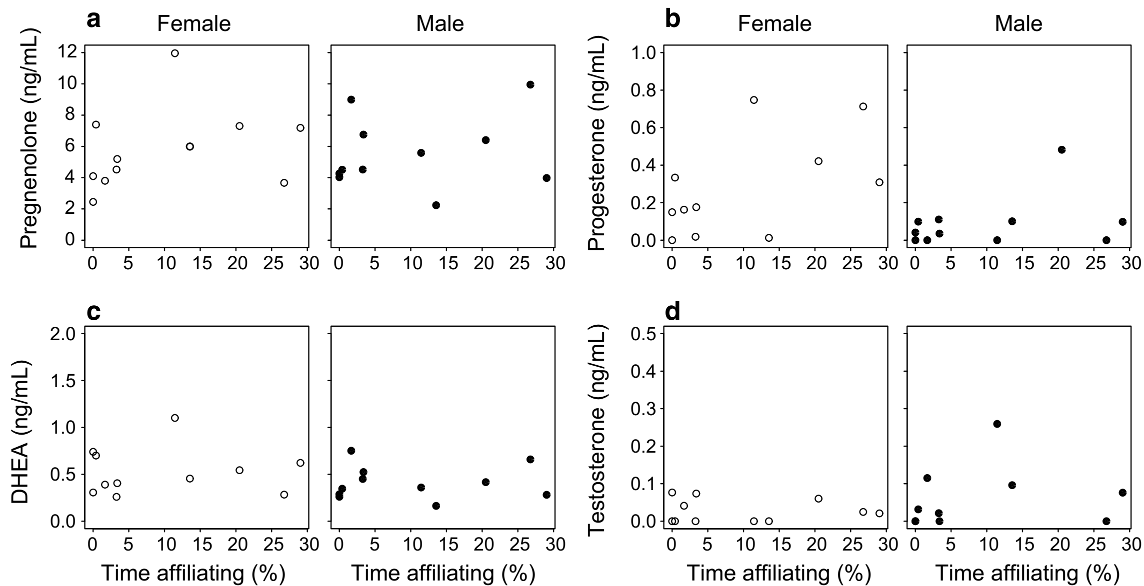

Fig. 3 Circulating steroid levels and affiliative behavior. Plasma steroid levels were averaged across timepoints for each individual. Time spent affiliating (clumping and co-nesting) was also averaged across timepoints for each pair. There were no significant correlations between plasma steroid levels and affiliative behavior: pregnenolone (**a**), progesterone (**b**), DHEA (**c**), and testosterone (**d**). Females data points are *open circles*, and male data points are *closed circles*

Surprisingly, we measured relatively high levels of circulating pregnenolone. While pregnenolone levels have been largely unstudied in songbirds, the circulating concentrations of pregnenolone in Japanese quail (*Coturnix japonica*) and red jungle fowl (*Gallus gallus*) are lower than what we report here (Tsutsui and Yamazaki 1995; Ericsson et al. 2014). Circulating levels of pregnenolone were correlated with circulating levels of DHEA (possibly an adrenal steroid) but not testosterone (a gonadal steroid). This pattern suggests that pregnenolone is secreted primarily by the adrenals. Interestingly, Ericsson et al. (2014) compared pregnenolone levels (measured with LC–MS/MS) in domesticated chickens and red jungle fowl and found higher levels in domesticated chickens (~5 vs. ~1 ng/mL). Future work could compare pregnenolone in domesticated and non-domesticated zebra finches.

Taken together, low circulating sex steroid levels in these finches in combination with relatively high circulating pregnenolone levels raises questions about local steroidogenesis in tissues such as the brain (Vanson et al. 1996). In other songbirds, brain synthesis of androgens and estrogens, either de novo from cholesterol or from a circulating sex steroid precursor, is higher when circulating sex steroids are extremely low (i.e., during non-breeding periods) (Schmidt et al. 2008; Pradhan et al. 2010). Future studies should examine the potential role of circulating pregnenolone as a prohormone in wild and domesticated zebra finches.

The backdoor pathway to 5α-DHT

We had predicted circulating ADIOL would be elevated if the backdoor pathway was being used, based on observations from tammar wallaby pouch young (Auchus 2004). ADIOL is secreted by the pouch young testes and travels to the skin and urogenital tract, where it is locally converted to 5α-DHT, when circulating testosterone is low (Auchus 2004). During this time, plasma levels of ADIOL are higher in males than females (Shaw et al. 2000).

We saw low plasma levels of testosterone in both female and male finches; however, we saw little evidence that 5α-reduced steroids in the backdoor pathway were present in circulation. Only a small number of plasma samples had detectable androsterone levels. Nonetheless, we cannot rule out the possibility that this pathway is used locally in the zebra finch brain for 5α-DHT synthesis, and future work should explore this possibility.

Evidence of progestins and androgens regulating pair bonding in zebra finches

Classically, progesterone is considered to be a reproductive steroid in females. However, progesterone is also important for the expression of both female and male parental behavior and may reflect pairing status in male birds (reviewed in: Lynn 2015). Furthermore, there is also evidence that it may regulate pairing behavior. Smiley et al. (2012) found that progesterone treatment to female zebra finches promoted courtship and nesting behaviors when administered prior to pairing, however progesterone treatment had no effect on pair-maintenance behavior when administered after pair-bond formation. Consistent with this, we found no relationship between male or female progesterone levels and pair-maintenance behavior (time spent affiliating). However, we did not look during pair-bond formation, and it is possible there may have been a relationship during that time.

Research examining the regulatory role of testosterone in pair bonding has largely focused on male song associated with courtship. After initial bond formation (~2 weeks), directed and undirected male songs also function as affiliative behaviors associated with bond maintenance and appear to synchronize breeding throughout the year (Dunn and Zann 1996; Zann 1996). However, male song is not necessary for pair-bond maintenance (Tomaszycki and Adkins-Regan 2006). While there is extensive work highlighting the importance of testosterone in the development and regulation of male song (Arnold 1975; Williams et al. 2003; Remage-Healey et al. 2009), the regulatory role of testosterone on male song associated with bond maintenance has not been directly examined.

The evidence for a role of androgens in regulating non-song pairing behavior is also mixed. Administration of flutamide (an anti-androgen) and ATD (an aromatase inhibitor) in male zebra finches decreased male aggression and female approaches during pair-bond formation and courtship, but had no effect on any other courtship behaviors (Tomaszycki et al. 2006). There have only been a few studies examining the role of sex steroids in zebra finch pair-bond maintenance (review: Prior and Soma 2015). Here, our results are difficult to interpret because circulating testosterone levels were very low in both males and females. We saw no relationship between circulating testosterone and affiliation associated with pair-bond maintenance.

Conclusions

The zebra finch is an extensively used animal model for neuroscience and behavioral studies, and yet surprisingly little is known about the physiology of wild or non-domesticated zebra finches. This is one of the first studies to use LC–MS/MS to examine steroid profiles of songbirds. While we found no evidence that sex steroids are involved in regulating pair-maintenance behavior in non-domesticated zebra finches, our data raise several interesting questions about

the neuroendocrinology of domesticated and non-domesticated zebra finches. The combination of low circulating testosterone levels and high circulating pregnenolone levels in non-domesticated zebra finches raises interesting questions about (1) the effect of domestication on zebra finch sex steroid profiles and HPG function, and (2) the role of elevated pregnenolone in both males and females.

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Compliance with ethical standards

Animal welfare statement All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

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