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Sex steroid profiles in zebra finches: Effects of reproductive state and domestication

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ABSTRACT

The zebra finch is a common model organism in neuroscience, endocrinology, and ethology. Zebra finches are generally considered opportunistic breeders, but the extent of their opportunism depends on the predictability of their habitat. This plasticity in the timing of breeding raises the question of how domestication, a process that increases environmental predictability, has affected their reproductive physiology. Here, we compared circulating steroid levels in various "strains" of zebra finches. In Study 1, using radioimmunoassay, we examined circulating testosterone levels in several strains of zebra finches (males and females). Subjects were wild or captive (Captive Wild-Caught, Wild-Derived, or Domesticated). In Study 2, using liquid chromatography–tandem mass spectrometry (LC–MS/MS), we examined circulating sex steroid profiles in wild and domesticated zebra finches (males and females). In Study 1, circulating testosterone levels in males differed across strains. In Study 2, six steroids were detectable in plasma from wild zebra finches (pregnenolone, progesterone, dehydroepiandrosterone (DHEA), testosterone, androsterone, and 5α-dihydrotestosterone (5α-DHT)). Only pregnenolone and progesterone levels changed across reproductive states in wild finches. Compared to wild zebra finches, domesticated zebra finches had elevated levels of circulating pregnenolone, progesterone, DHEA, testosterone, androstenedione, and androsterone. These data suggest that domestication has profoundly altered the endocrinology of this common model organism. These results have implications for interpreting studies of domesticated zebra finches, as well as studies of other domesticated species.

1. Introduction

Natural selection shapes both the behavior of wild animals and its underlying physiological mechanisms. The process of domestication alters natural selection and introduces artificial selection pressures. Some effects of domestication are similar across species (Zeder, 2012; Wilkins et al., 2014). For example, domesticated animals show changes to the seasonal regulation of reproduction and molt (Belyaev, 1979; Lincoln et al., 1990; Setchell, 1992). Furthermore, domesticated animals show decreased hypothalamic–pituitary–adrenal (HPA) axis reactivity (Martin, 1978; Woodward and Strange, 1987; Künzl and Sachser, 1999; Albert et al., 2008; Suzuki et al., 2012; Homberger et al., 2013; Ericsson et al., 2014). However, other effects of domestication are different across species and depend on the characteristics of the original population and the specifics of the domestication process (Zeder, 2012; Adkins-Regan, 2009).

Zebra finches are an extensively used model organism in a wide range of behavioral, evolutionary, and neuroscience research (Griffith and Buchanan, 2010). Initial breeding stocks of zebra finches were removed from the wild in Australia during the 19th century and were established into a number of independent breeding populations across the globe (Zann, 1996). Given the ease with which they can be studied in the laboratory, it is not surprising that most of the research on the physiology and neuroscience of this species has been conducted on domesticated zebra finches

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(Griffith and Buchanan, 2010). The laboratory environment itself may have caused persistent differences to arise between wild and domesticated individuals with respect to both their physiology and behavior (Calisi and Bentley, 2009; Dickens and Bentley, 2014). Furthermore, the process of domestication may have led to genetic differentiation between wild and domesticated zebra finch populations, even in the few hundred generations for which they have been separated (Forstmeier et al., 2007). It is currently unclear to what extent there may be population differences in functional traits relating to behavior and physiology, between wild and domesticated finches and also across different domesticated populations. The demonstration of such effects will provide insight into the mechanisms underlying domestication, but may also necessitate caution when comparing across populations of zebra finches that differ in their domestication history.

For some traits, wild and domesticated zebra finches are extremely similar. For example, both wild and domesticated zebra finches form long-term pair bonds, provide biparental care, and breed opportunistically (Zann, 1996). With regard to morphology, zebra finches with wild-type plumage are common in domesticated populations, and beak coloration is similar in wild and domesticated zebra finches (Burley et al., 1992). However, other traits differ between wild and domesticated zebra finches. Domesticated zebra finches are generally larger and express many different plumage colorations (Sossinka, 1970; Carr and Zann, 1986; Zann, 1996; Forstmeier et al., 2007). Furthermore, wild zebra finches have lower rates of extra-pair copulations (Griffith et al., 2010) and differ subtly in nesting behavior (Mainwaring et al., 2010; Gilby et al., 2013). In mate choice tests, captive wild-caught females are choosier than domesticated females, and domesticated males are more active courters than wild-caught males (Rutstein et al., 2007). However, it is currently unclear whether there are fundamental differences between wild and domesticated zebra finches in their endocrine profiles.

Zebra finches are opportunistic breeders; however, the extent of opportunism varies with habitat and environmental conditions (Zann, 1996; Perfito et al., 2007). Overall, the regulation of breeding in zebra finches is not as well understood as it is in seasonally-breeding species. However, studies have suggested that the hypothalamic–pituitary–gonadal (HPG) axis functions similarly in wild and domesticated zebra finches (Perfito et al., 2007, 2011; Prior and Soma, 2015). For example, the effects of natural droughts on wild finches and the effects of experimental water restriction on domesticated finches are similar (Perfito, 2010; Prior and Soma, 2015). Both cause zebra finches to stop breeding and inhibit parts of the HPG axis (Prior et al., 2013). Therefore, it is likely that (1) wild zebra finches have sex steroid profiles that change across a breeding cycle; and that (2) domestication, a process that reduces environmental variability, has affected sex steroid profiles.

We began to investigate the above hypotheses in two studies. In Study 1, we used a radioimmunoassay (RIA) to quantify circulating testosterone levels in males and females from four "strains" of zebra finches: Wild, Captive Wild-Caught, Wild-Derived (males only), and Domesticated. In Study 2, we used liquid chromatography–tandem mass spectrometry (LC–MS/MS) to quantify multiple sex steroids and precursors in wild and domesticated zebra finches. We also described circulating steroid profiles of wild zebra finches at different reproductive states across a breeding cycle.

2. Materials and methods

2.1. Ethics statement

These studies were carried out under a UBC Animal Care Committee protocol and under ethics licenses from the Animal Ethics Committee at Deakin University (B24-2012) and Macquarie University (ARA 2010/053). Experiments followed the guidelines of the Canadian Council on Animal Care and the Australian and New Zealand Council for the Care of Animals in Research and Teaching.

2.2. Study 1: radioimmunoassay (RIA) to measure testosterone levels in plasma

We used a radioimmunoassay (RIA) to compare circulating testosterone levels among four strains of zebra finches (1) Wild ($n = 20$ females and 11 males), (2) Captive Wild-Caught ($n = 4$ females and 4 males), (3) Wild-Derived ($n = 0$ females and 42 males), and (4) Domesticated ($n = 15$ females and 15 males).

Wild finches were sampled while "not nesting" or while actively nesting (Incubating or Feeding Chicks) (females: $n = 8$ Not Nesting and 12 Nesting; males: $n = 5$ Not Nesting and 6 Nesting). Subjects in other strains were not nesting at the time of blood sampling, allowing us to compare circulating testosterone levels across all four strains, in subjects that were not nesting. Wild-Derived males were housed in same-sex aviaries, and all other subjects were paired. See below for more details.

2.3. Subjects

2.3.1. Wild zebra finches

Free-roaming, wild zebra finch pairs were captured for blood sampling at Fowler's Gap Arid Zone Research Station, in the Western region of New South Wales, Australia. More specifically, pairs were from nest box colonies within Gap Hills (Griffith et al., 2008). Blood samples were collected between September–November 2012. At this location, October is often a peak breeding month for zebra finches (Griffith et al., 2008), and in this season of 2012, there was continuous breeding activity in the study area throughout the time of sampling. However, in other areas of the Fowler's Gap study site, environmental conditions were different and there was no breeding.

At the time of blood sampling, subjects were either not nesting or nesting. Breeding activity in nest box populations was assessed every 1–3 days. For active nests, the dates that eggs were laid and hatched were recorded, allowing us to determine the days of Incubation and Feeding Chicks. Breeding females and males (either Incubating or Feeding Chicks) were caught in the nest box during the early morning. Subjects that were not nesting were caught in a walk-in feeder trap from an area where no birds were nesting in nest boxes (Mariette et al., 2011; McCowan et al., 2015). While there was no nesting at these sites, because the overall environmental conditions were appropriate for breeding, it is difficult to be certain that these individuals were not maintaining a breeding nest elsewhere. Therefore, we classified these individuals as not nesting, instead of non-breeding, because they may have been physiologically capable of breeding (Prior et al., 2013; Perfito et al., 2007). Note that in zebra finches, only females develop a brood patch (Zann, 1996). Additionally, all individuals examined in these colonies had a brood patch score of 1–2, making it difficult to identify breeding state from brood patch score.

2.3.2. Captive Wild-Caught zebra finches

Wild adult female and male zebra finches were caught at the Fowler's Gap study site (in September 2012) and housed in open-air aviaries under natural photoperiod for 1–2 months prior to collecting blood samples. Subjects had access to *ad libitum* seed, water, cuttlefish bone, and grit. These subjects were housed in several aviaries, and each aviary held 1–2 pairs. All subjects were pair bonded; however, they were not nesting at the time of blood sampling (Prior et al., 2016).

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2.3.3. Wild-Derived zebra finches

Wild-Derived adult male zebra finches were also examined. These subjects were largely first- or second-generation and born in captivity at Macquarie University. Some of these birds might be the original individuals taken from the wild in either 2007 or 2009 (from Fowler's Gap and another population in Far-Western New South Wales). These subjects were housed in a same-sex open-air aviary ($8 \times 10 \times 2$ m). All individuals had *ad libitum* access to seed, water, cuttlefish bone, and grit. Blood was collected in the morning. Prior to blood collection, individuals were housed in the aviary for one month without contact with females. Therefore, these males were not nesting.

2.3.4. Domesticated zebra finches

Adult zebra finches (>120 days old) housed at University of British Columbia (UBC) were used as the domesticated sample. This colony was derived from birds from Eastern Birds Supplies (Quebec, Canada) and birds from Simon Fraser University (Burnaby, Canada) (Forstmeier et al., 2007). At UBC, subjects were housed indoors at ~22 °C, ~35% relative humidity, and on a 14:10 h light:-dark cycle. All individuals had *ad libitum* access to seed (50/50, panicum millet/white millet, Just For Birds, Langley BC), water, cuttlefish bone, and grit. These birds were housed in pairs, which were not nesting.

2.4. Blood collection

We collected 100–150 μL of blood from the brachial vein. Time to bleed after capture by Strain: Wild = within 15 min (7.3 ± 0.3 min); Captive Wild-Caught = within 13 min (6.9 ± 0.4 min); Wild-Derived = within 7 min (3.1 ± 0.2 min); Domesticated = within 10 min (4.0 ± 0.1 min). For Wild zebra finches, blood samples were kept on wet ice until centrifugation to obtain plasma. For all other strains, blood samples were immediately centrifuged. All plasma samples were frozen until steroid quantification via RIA.

2.5. RIA to measure plasma testosterone levels

Steroids were extracted from plasma samples (10–20 μL) using solid phase extraction (Newman et al., 2008; Taves et al., 2010, 2011). Testosterone was quantified using a sensitive and specific RIA (MP Biomedicals, 07189102). Validations and modifications of this RIA have been described (Overk et al., 2013). Samples were analyzed in 4 assays. Intra-assay variation was 10.76% and inter-assay variation was 12.75%. Samples were corrected for recovery of testosterone from zebra finch plasma (102.5%).

2.6. Study 2: liquid chromatography–tandem mass spectrometry (LC–MS/MS) to measure sex steroid profiles in plasma

We used a sensitive and specific liquid chromatography–tandem mass spectrometry (LC–MS/MS) method to quantify circulating steroids. Wild zebra finch samples were collected as described above during September–November 2012. Here samples were categorized into 3 breeding stages: Not Nesting ($n = 11$ females and 10 males), Incubating ($n = 17$ females and 7 males), and Feeding Chicks ($n = 9$ females and 7 males). Some of these individuals are the same as the wild zebra finches in Study 1.

In addition, steroid profiles were compared between wild zebra finches ($n = 37$ females and 24 males) and domesticated zebra finches ($n = 8$ females and 5 males). Again, UBC domesticated zebra finches were sampled. These are different individuals than those sampled for Study 1, although all birds were housed under the same conditions (see above). For this comparison, both wild and domesticated finches were not nesting. Blood sampling was the same as above.

2.7. LC–MS/MS to measure plasma steroid profiles

Steroid extraction and LC–MS/MS protocols were adapted from Kalhorn et al., 2007. This LC–MS/MS assay is optimized to measure 11 androgens and progestins: 6 steroids from the "traditional" pathway to 5α-dihydrotestosterone (5α-DHT) [pregnenolone; progesterone; 17-OH-pregnenolone; dehydroepiandrosterone (DHEA); androstenedione and testosterone], 4 steroids from the "backdoor" pathway to 5α-DHT [pregnan-3,20-dione; pregnan-3α,ol-20-one; pregnan-3,17-diol-20-one and androsterone], and 5α-DHT (Auchus, 2004; Fokidis et al., 2015; Prior et al., 2016).

Internal standards (deuterated testosterone, 5α-DHT and androstanediol, C/D/N Isotopes Inc, Quebec Canada) were added to plasma samples (~30 μl). Steroids were then extracted using 2000 μl of 60:40 (v/v) hexane:ethyl acetate for 30 min, and the upper layer was collected. Next, two additional extractions were done and pooled with the initial extract to maximize extraction efficiency. Pooled extracts were dried in a centrifugal vacuum evaporator (Centrivap, Labconco).

The resulting residues were derivatized using hydroxylamine (HA, Fluka, Sigma, Oakville Ontario Canada), which enhances sensitivity for ketosteroids. Extracts were dissolved in 50 μL of 50 mM HA in 50% methanol, centrifuged at 20,000*g* for 2 min, transferred to an LC insert, and then heated to 65 °C for 30 min prior to LC–MS/MS analysis.

Analysis was carried out with the analytical system of a Waters Acquity UPLC Separations Module coupled to a Waters Quattro Premier XE Tandem Mass Spectrometer (Waters Corporation, Massachusetts, USA). A 2.1×100 mm BEH 1.7 μM C18 column was used (Water Corporation, Massachusetts, USA). The two mobile phases were water and acetonitrile, both containing 0.1% formic acid, using the following gradient: 0 min, 25%; 0.2 min, 25%; 8 min, 70%; 9 min, 100%; 10 min, 100%; 10.2 min, 25% (% acetonitrile) with a total run length of 12 min. Column temperature was 35 °C and injection volume was 15 μL. The MS was set at unit resolution, capillary was 3 kV, source and desolvation temperatures were 120 °C and 300 °C respectively, desolvation and cone gas flows were 1000 L/h 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 (MRM) with instrument parameters optimized for the m/z ratios and corresponding fragments of the oxime-steroids monitored for each MRM.

Data processing was conducted with Quanlynx (Waters Corporation Massachusetts, USA) using area under curve of analyte/IS. Calibration samples consisted of neat standards and equivalently extracted spiked charcoal-stripped serum (6 standards ranging from 0.02 to 10 ng/ml) and normalized to sample volume. The limit of detection (LOD) ranged from approximately 0.01–0.02 ng/mL (3x background). Recoveries and conversions to derivatized steroid species were greater than 80% for each steroid. Statistics were conducted for steroids where some samples were above the LOD; for these steroids, all samples below the LOD were set to zero.

2.8. Statistics

All statistics were conducted in R v3.1.2 (R core team, 2014). Time to bleed did not significantly impact circulating steroid levels, and thus no such correction was made in the models. ANOVAs were run using glm models, and data were transformed as necessary to meet the assumptions of the test. All models are reported as ANOVA summaries with the resulting *F* statistic.

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2.8.1. Study 1: RIA to measure testosterone levels in plasma

In order to test whether Sex or Nesting Activity (Not Nesting vs. Nesting) affected circulating testosterone levels of Wild zebra finches, we used an ANOVA with Sex and Nesting Activity as between-subject factors.

In zebra finches that were not nesting, in order to test whether Strain (Wild, Captive Wild-Caught, Wild-Derived, and Domesticated) affected circulating testosterone levels, we used a non-parametric one-way ANOVA (Kruskal–Wallis test), with Strain as a between-subject factor. Note that no Wild-Derived females were sampled. Separate Kruskal–Wallis tests were conducted on females and males. Pair-wise comparisons were conducted using Wilcoxon rank sum tests.

2.8.2. Study 2: LC–MS/MS to measure sex steroid profiles in plasma

In order to test whether sex steroid levels varied with Reproductive State in wild finches, we conducted an ANOVA for each steroid. Sex and Reproductive State (Not Nesting, Incubating, or Feeding Chicks) were between-subject factors. Significant main effects of Reproductive State were followed up with pair-wise Wilcoxon rank sum tests.

In zebra finches that were not nesting, in order to test whether sex steroids differed between wild and domesticated finches, we conducted an ANOVA for each steroid. Sex and Strain (Wild or Domesticated) were both between-subject factors. Significant interactions were followed up with Wilcoxon rank sum tests (Strain) for females and males separately.

3. Results

3.1. Study 1: RIA to measure testosterone levels in plasma

In Wild zebra finches, there was no effect of Nesting Activity (Not Nesting vs. Nesting) on female or male circulating testosterone levels (Fig. 1A; $F_{1,28} = 0.24$, $P = 0.63$). There was also no Sex × Nesting Activity interaction (Fig. 1A; $F_{1,27} = 0.35$, $P = 0.56$). However, there was a main effect of Sex, with males having significantly higher circulating testosterone levels than females (Fig. 1A; $F_{1,29} = 8.30$, $P < 0.01$).

We then focused on subjects that were Not Nesting in 4 "strains" (Wild, Captive Wild-Caught, Wild-Derived, and Domesticated). The effect of Strain was examined in females and males separately. Overall, circulating testosterone varied significantly by Strain in females (Fig. 1B; Kruskal–Wallis, $\chi^2 = 15.22$, $P < 0.001$) and males (Fig. 1C; $\chi^2 = 30.71$, $P < 0.001$). In females, Wild females had higher circulating testosterone than Captive Wild-Caught females and Domesticated females (Fig. 1B; $P \leqslant 0.01$ in both cases). In males, Wild and Domesticated males had higher circulating testosterone than Wild-Derived males (Fig. 1C; $P < 0.001$ in both cases).

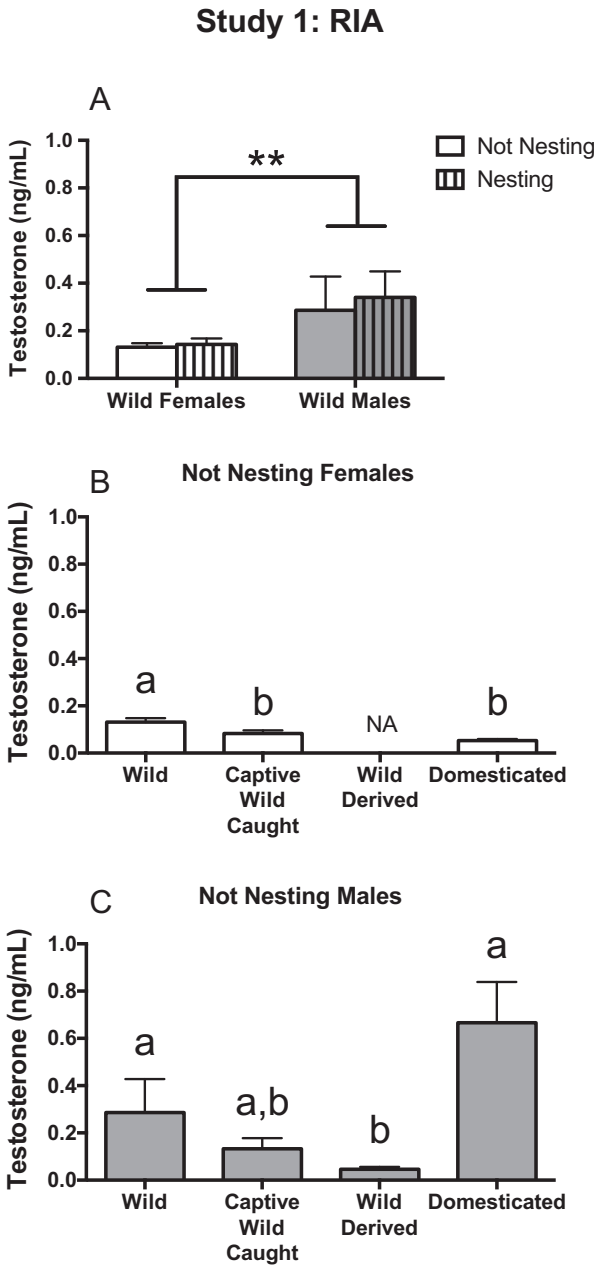

Fig. 1. In Study 1, plasma testosterone levels were measured via radioimmunoassay (RIA). (A) Plasma testosterone levels in Wild zebra finches did not vary with Nesting Activity but were higher in males than females. Here, Nesting zebra finches includes subjects that were Incubating or Feeding Chicks. (B) In female zebra finches that were Not Nesting, plasma testosterone levels differed across "strains" (Wild, Captive Wild-Caught, and Domesticated) and were highest in Wild females. Wild-derived females were not assessed (NA). (C) In male zebra finches that were Not Nesting, plasma testosterone levels also differed across strains and were lowest in Wild-Derived males in captivity. In (A), $^{**}p \leqslant 0.01$. In (B) and (C), letters indicate significant differences.

3.2. Study 2: LC–MS/MS to measure sex steroid profiles in plasma

Six steroids were detectable in the circulation of wild zebra finches: pregnenolone, progesterone, DHEA, testosterone, androsterone, and 5α-DHT (Fig. 2; Table 1). However, androsterone and 5α-DHT were only detectable in a few plasma samples (Table 1). Androstenedione was not detectable in plasma samples from wild zebra finches.

Levels of pregnenolone, progesterone, and DHEA were not significantly different between females and males (pregnenolone: $F_{1,57} = 2.58$, $P = 0.11$; progesterone: $F_{1,57} = 0.16$, $P = 0.69$; DHEA: $F_{1,57} = 2.63$, $P = 0.11$; Fig. 3A–C). Testosterone levels were significantly higher in males than females ($F_{1,57} = 8.31$, $P < 0.01$; Fig. 3D).

Pregnenolone and progesterone levels differed across Reproductive State (pregnenolone: $F_{2,58} = 4.59$, $P = 0.01$; progesterone: $F_{2,58} = 10.67$, $P < 0.001$; Fig. 3A and B). Pregnenolone levels were highest during Feeding Chicks (Fig. 3A; Not Nesting vs. Feeding Chicks, $P = 0.03$; Incubating vs. Feeding Chicks, $P = 0.04$; Not Nesting vs. Incubating, $P = 0.94$). Similarly, progesterone levels were highest during Feeding Chicks and lowest during Not Nesting (Fig. 3B; Not Nesting vs. Feeding Chicks, $P < 0.001$; Incubating vs. Feeding Chicks, $P < 0.01$; Not Nesting vs. Incubating, $P < 0.01$). Circulating DHEA levels (Fig. 3C) and testosterone levels (Fig. 3D)

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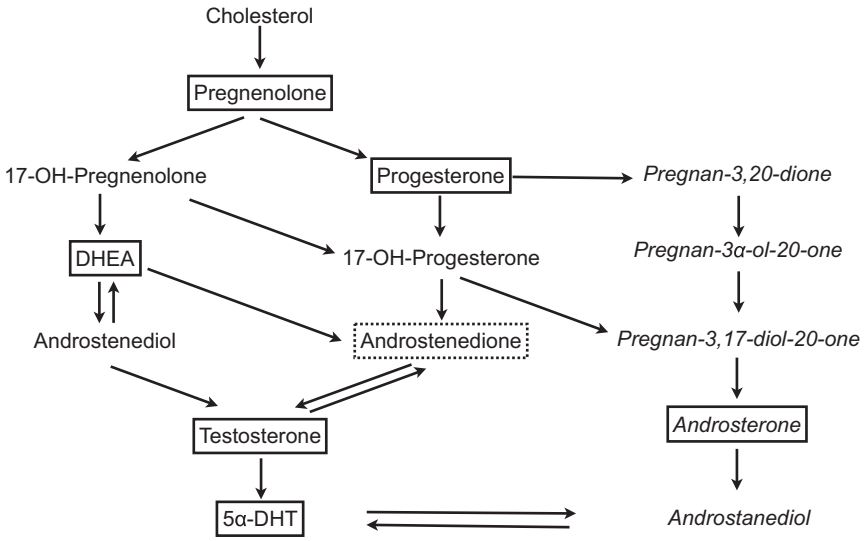

Fig. 2. A simplified steroidogenic pathway indicating steroids in the "traditional" pathway to 5α-DHT and the "backdoor" pathway to 5α-DHT. Steroids in the backdoor pathway are in italics. Boxed steroids indicate those that were detectable in the plasma of zebra finches (solid line = detectable in Wild and Domesticated finches; dotted line = detectable in Domesticated finches only).

Table 1
Percent of plasma samples from Wild and Domesticated zebra finches that are non-detectable (ND), 3–10X above background, or >10X above background using a LC–MS/MS method to simultaneously examine multiple steroids.

		Pregnenolone	Progesterone	DHEA	Androsterone	Androstenedione	Testosterone	5α-DHT
Wild $n = 61$	ND	0	13	0	61	0	54	93
	3–10X	0	41	0	37	0	26	0
	>10X	100	46	100	2	0	20	7
Domesticated $n = 13$	ND	0	0	0	38	46	0	70
	3–10X	0	30	0	54	38	30	30
	>10X	100	70	100	8	15	70	0

Note: Both males and females are included here. Wild zebra finches were in several Reproductive States, and Domesticated zebra finches were sampled while Not Nesting.

were not affected by Reproductive State in males or females (DHEA: $F_{2,58} = 1.77$, $P = 0.18$; testosterone: $F_{2,58} = 0.93$, $P = 0.40$). There were no significant Sex × Reproductive State interactions (all P values > 0.05). Because the residuals of the GLM model for testosterone were not normally distributed, we confirmed there was no effect of Reproductive State using Kruskal–Wallis tests (Fig. 3D; females: $\chi^2 = 1.39$, $P = 0.50$. males: $\chi^2 = 0.65$, $P = 0.72$).

Independent of Reproductive State, Domesticated zebra finches had a greater number of steroids that were detectable in plasma, compared to Wild zebra finches. First, 7 steroids were detectable in the circulation of Domesticated zebra finches (Table 1; Fig. 2). Androstenedione, which was not detectable in plasma from Wild zebra finches, was detectable in plasma from Domesticated males and females. Second, a greater proportion of Domesticated zebra finch samples had detectable levels of progesterone, testosterone, androsterone, and 5α-DHT (Table 1).

Next, we focused on subjects that were Not Nesting and compared Wild and Domesticated zebra finches. For the 6 steroids that were detectable in the circulation of both Wild and Domesticated zebra finches, steroid levels were generally higher in Domesticated finches than wild finches (Table 2, Fig. 4A–G). Circulating levels of testosterone were higher in males than females, regardless of Strain (Table 2, Fig. 4D).

We further tested the effect of Strain on androsterone and 5α-DHT separately in males and females (Fig. 4F–G). Domesticated males had higher circulating androsterone than Wild males (Fig. 4F; $W = 49.0$; $P < 0.01$), but this pattern was not significant for females (Fig. 4F; $W = 55.5$, $P = 0.24$). Circulating 5α-DHT was not significantly different between Wild and Domesticated zebra finches (Fig. 4G; males, $W = 38.0$; $P = 0.07$; females, $W = 49.5$, $P = 0.29$).

4. Discussion

Our results highlight the effects of both Strain (level of domestication) and Reproductive State on circulating steroid profiles of male and female zebra finches. In Study 1, we found significant main effects of level of domestication on circulating testosterone levels of males and females that were Not Nesting. Domesticated males had significantly higher circulating testosterone levels relative to Wild-Derived males. While the direction of this effect was not consistent across the sexes in Study 1, in Study 2, both male and female circulating testosterone levels were significantly elevated in Domesticated zebra finches, relative to Wild zebra finches. Furthermore, in Study 2, we found that circulating levels of several steroids were consistently elevated in Domesticated zebra finches. During domestication, selection may have acted on traits relating to mate acquisition and resource defense in a captive environment, which could have contributed to this result. In Study 2, we also found that circulating pregnenolone and progesterone levels varied across the Reproductive States in Wild zebra finches. To our knowledge, this is the first characterization of steroid profiles in wild zebra finches. Finally, from our literature review, we conclude that large variation across strains and reproductive states is a phenomenon seen across multiple laboratories that have studied zebra finches.

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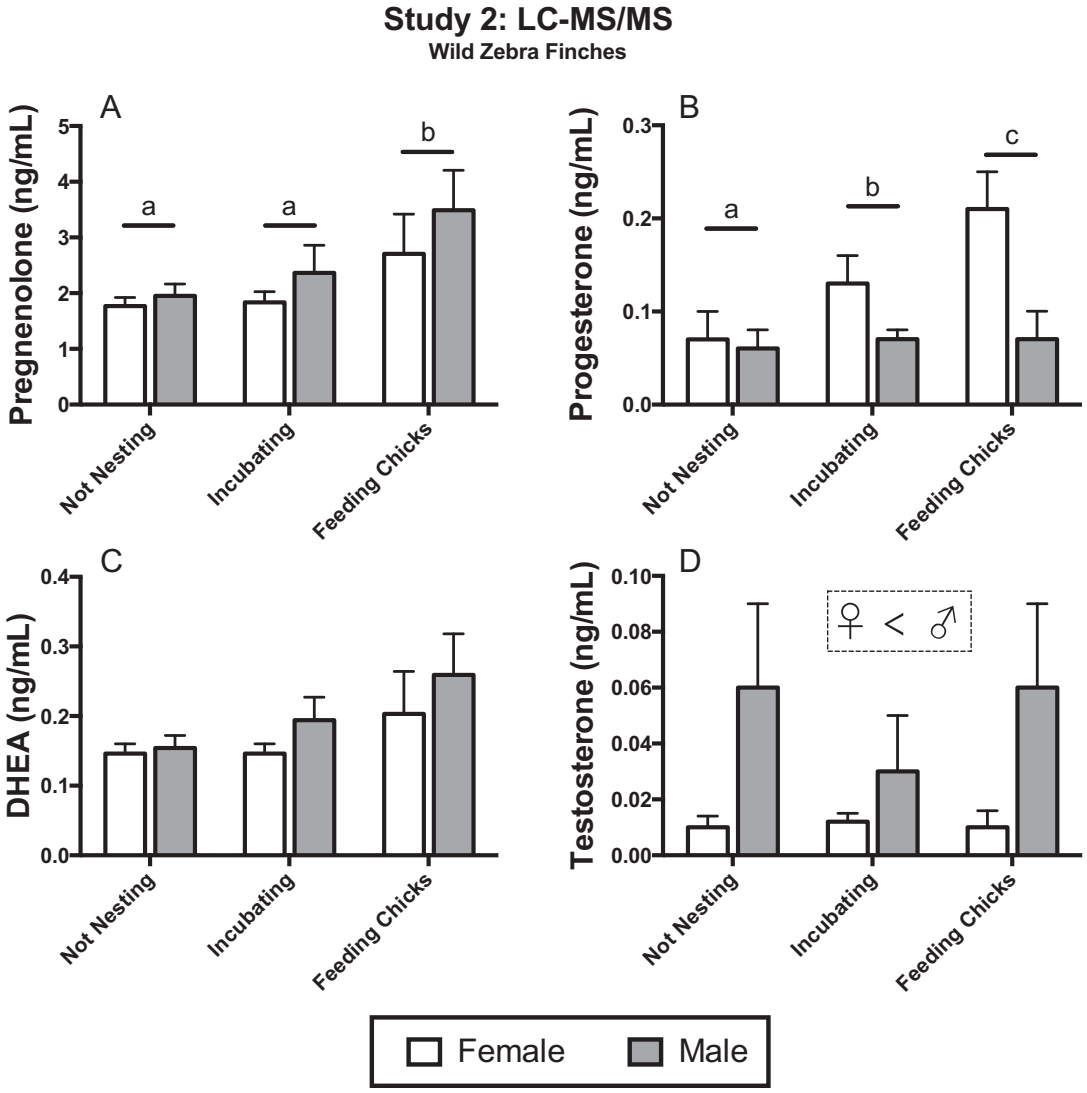

Fig. 3. In Study 2, a panel of steroids was measured via liquid chromatography–tandem mass spectrometry (LC–MS/MS) in plasma from Wild zebra finches. Wild female and male zebra finches were examined across Reproductive States. (A) Pregnenolone, (B) Progesterone, (C) DHEA, and (D) Testosterone. Plasma pregnenolone and progesterone levels were higher during the Feeding Chicks stage, and plasma testosterone levels were higher in males than females. In (A) and (B), letters indicate significant differences.

4.1. Steroid profiles in the circulation of wild and domesticated zebra finches

In Study 2, the observed elevations of both circulating steroid hormones and their precursors in domesticated zebra finches may signify a general elevation of all, or many, sex steroids in circulation and tissues. Such a general elevation could be caused by differences in the availability of the steroid precursor, cholesterol, or by differences in proteins involved in early stages of gonadal and adrenal steroidogenesis (e.g., TSPO, StAR, CYP11A1). Future studies should examine levels of cholesterol in the circulation and levels of steroidogenic proteins in the gonads and adrenals of wild and domesticated zebra finches.

In addition, the data indicate variation in circulating steroid levels across the breeding cycle. Many songbirds show peaks in circulating testosterone during courtship and/or egg laying (e.g., Vleck and Priedkalns, 1985; Hegner and Wingfield, 1986; Ball and Wingfield, 1987; Wingfield and Farner, 1978). Here, we found no changes in circulating testosterone levels across three reproductive states in wild zebra finches; however, all of our samples were collected after egg laying and we might have missed an earlier peak in testosterone. Interestingly, we did see elevations of circulating pregnenolone and progesterone during Feeding Chicks. To our knowledge, this pattern has not been previously reported in songbirds. The functions of pregnenolone and progesterone during

Table 2
Effects of strain (Wild or Domesticated) and Sex on circulating steroid levels, as measured by a LC/MS–MS method.

	Strain		Sex		Strain × sex	
	$F_{(1,32)}$	P	$F_{(1,31)}$	P	$F_{(1,30)}$	P
Pregnenolone	**96.18**	**<0.001**	<0.01	0.95	0.47	0.50
Progesterone	**31.86**	**<0.001**	0.19	0.67	1.84	0.19
DHEA	**44.05**	**<0.001**	1.53	0.23	3.35	0.08
Testosterone	**22.15**	**<0.001**	**13.97**	**<0.001**	0.11	0.75
Androsterone*	**20.84**	**<0.001**	**12.92**	**0.001**	**11.51**	**<0.01**
5α-DHT*	**4.33**	**<0.05**	0.26	0.61	0.06	0.80

Note: ANOVAs were run using glm models, and data were transformed as necessary to meet the assumptions of the test. All models are reported as ANOVA summaries with the resulting F statistic. All subjects were not nesting. Bolded values indicate significant effects.

* For androsterone and 5α-DHT, residuals of the GLM models were not normally distributed, and Wilcoxon rank sum tests were used to determine the effect of Strain on females and males separately.

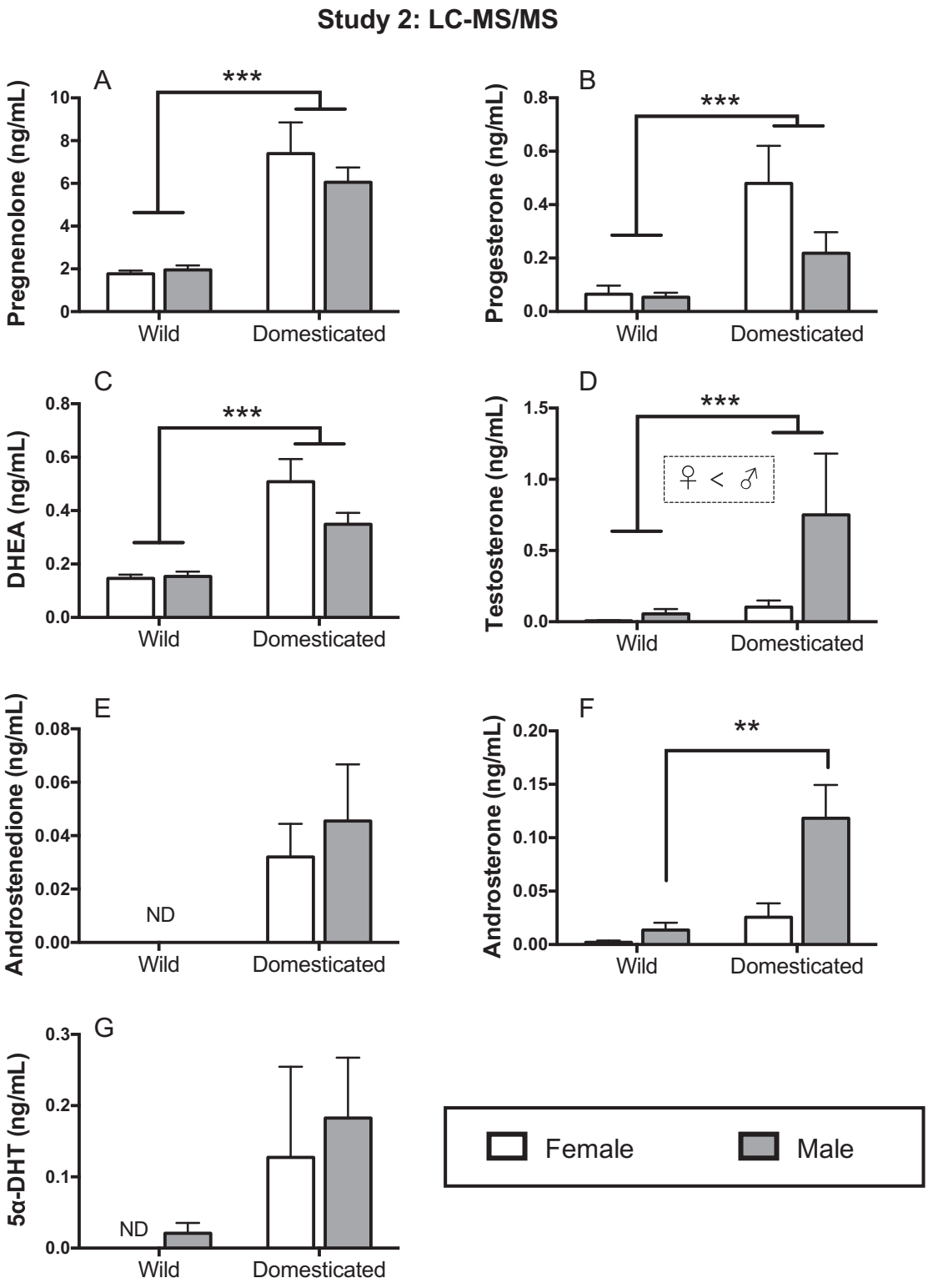

Fig. 4. In Study 2, a panel of steroids was measured via LC–MS/MS in plasma from Wild and Domesticated zebra finches that were Not Nesting. Overall, Domesticated zebra finches have higher circulating steroid levels than Wild finches. (A) Pregnenolone, (B) Progesterone, (C) DHEA, (D) Testosterone, (E) Androstenedione, (F) Androsterone, and (G) 5α-DHT. ND = non-detectable. $^{*}p \leqslant 0.05$, $^{**}p \leqslant 0.01$, $^{***}p \leqslant 0.001$.

Feeding Chicks are unclear. These steroids might regulate egg laying (Furr et al., 1973), and circulating levels of pregnenolone in Greylag geese (*Anser anser)* are lowest during incubation (Hirschenhauser et al., 1999).

Furthermore, we compiled circulating testosterone levels in male zebra finches across reproductive states from previously published studies (Table 3). Consistent with the present results, there is a general pattern of elevated plasma testosterone levels in males from domesticated populations of zebra finches compared to wild stocks, across reproductive conditions (Table 3). Additionally, there is pronounced variability in circulating testosterone levels across studies of domesticated males. Some of this variability likely

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Table 3
Studies reporting circulating testosterone levels in Wild or Domesticated adult male zebra finches.

	References	Wild vs. Domesticated	Housing conditions	Strain/location	Testosterone (ng/mL)	Method	*n*
Non-Breeding	Vleck and Priedkalns (1985)	Wild-Caught	Water Restricted	Barmera, S. Aus., AUS	<0.28*	RIA	16 (1 pooled)
	Priedkalns et al. (1984)	Wild-Caught	Water Restricted	S. Australia, AUS	<0.45*	RIA	16 (4 pooled)
	Perfito et al. (2007)	Wild	NA	Alice Springs, AUS	0.20 ± 0.02	RIA	2 (2/11 samples were detectable)
	Prior et al. (2013)	Domesticated	Water Restricted	Vancouver, BC, CAN	0.70 ± 0.2	RIA	11
Not Nesting	Hutchison et al. (1984)	Domesticated	Individually housed	Cambridge, UK	0.29	RIA	9
	Wade et al. (1996)	Domesticated	Same Sex housed	Los Angeles, CA, USA	0.52 ± 0.1	RIA	7
	Alonso-Alvarez et al. (2007)	Domesticated	Individually housed	Station Biologique de Foljuif, FR	1.58 ± 0.4	RIA	12
	Prior et al. (2013)	Domesticated	Not Nesting	Vancouver, BC, CAN	1.90 ± 0.4	RIA	9
	Charlier et al. (2010)	Domesticated	Same Sex housed	Vancouver, BC, CAN	2.50	RIA	8
	Lynn et al. (2015)	Domesticated	Individually housed	Berkeley, CA, USA	3.50	EIA	8
Breeding	Perfito et al. (2007)	Wild	NA	Victoria, AUS	0.51 ± 0.24	RIA	6 (6/8 samples were detectable)
	Vleck and Priedkalns (1985)	Domesticated	Late Incubating	Adelaide, S. Aus., AUS	0.24–1.06*	RIA	5 (1 pooled)
	Vleck and Priedkalns (1985)	Domesticated	Feeding Chicks	Adelaide, S. Aus., AUS	0.25*	RIA	4 (1 pooled)
	Koren et al. (2012)	Domesticated	?	Vancouver, BC, CAN	0.51	LC–MS/MS	7
	Vleck and Priedkalns (1985)	Domesticated	Courting	Adelaide, S. Aus., AUS	0.80*	RIA	9 (1 pooled)
	Vleck and Priedkalns (1985)	Domesticated	Early Incubating	Adelaide, S. Aus., AUS	1.06*	RIA	4 (1 pooled)
Unknown	Vleck and Priedkalns (1985)	Wild-Caught	?	Barmera, S. Aus., AUS	0.30*	RIA	5 (1 pooled)
	Wilbrecht et al. (2006)	Domesticated	?	Millbrook, NY, USA	0.16 ± 0.16	RIA	4
	Korsia and Bottjer (1991)	Domesticated	?	Los Angeles, CA, USA	0.17 ± 0.13	RIA	13
	Wilbrecht et al. (2006)	Domesticated	?	Millbrook, NY, USA	0.25 ± 0.1	RIA	6
	Schlinger et al. (1999)	Domesticated	?	Los Angeles, CA, USA	0.31	RIA	?
	Pröve (1983)	Domesticated	?	Bielfeld, Germany	0.33	RIA	7
	Luine et al. (1980)	Domesticated	?	Deer Park, NY, USA	0.65 ± 0.1	RIA	9
	Wilbrecht et al. (2006)	Domesticated	?	Millbrook, NY, USA	0.68 ± 0.37	RIA	5
	Adkins-Regan et al. (1990)	Domesticated	?	College Park, MD, USA	1.90	RIA	16
	Vockel et al. (1990)	Domesticated	?	Liege, Belgium	3.20	RIA	16
	Kabelik et al. (2011)	Domesticated	?	Bloomington, IN, USA	3.47 ± 0.67	RIA	6

Note: If we estimated testosterone levels from graphs, then we present only the estimated mean. If the mean and SEM were reported in the text of the original reference, then we provide those here. The strain/location of the last author is reported when no specific details are listed in the text. NA = not applicable, EIA = enzyme immunoassay, RIA = radioimmunnoassay, LC–MS/MS = liquid chromatography–tandem mass spectrometry.

* Total androgens measured, not a specific assay for testosterone.

reflects different assay methods, but some of the variability might reflect genetic and environmental differences among domesticated populations. Taken together, these results highlight the importance of reporting and controlling for reproductive state and population in studies of zebra finches. Future studies should examine circulating testosterone levels across a full breeding cycle (including non-breeding condition) for both wild and domesticated zebra finches.

4.2. Environmental predictability and breeding in zebra finches

While zebra finches have long been cited as the classic example of an opportunistically-breeding bird species (Serventy, 1971; Perfito et al., 2007; Hahn et al., 2008; Griffith and Buchanan, 2010), the degree of this opportunism depends on the predictability of the habitat. Zebra finches breed more seasonally along the coast of Australia, where rain and weather conditions are seasonally predictable (Perfito, 2010). Perfito et al. (2007) compared the hypothalamic–pituitary–gonadal (HPG) axis of non-breeding (winter) zebra finches from the coast and non-breeding (drought) zebra finches from central Australia. Non-breeding zebra finches from the predictable coastal habitat had lower circulating levels of luteinizing hormone (LH) and smaller gonads than non-breeding zebra finches from the unpredictable central habitat (Perfito et al., 2007). It is possible that in unpredictable environments, zebra finches maintain intermediate levels of circulating testosterone year round.

Assuming captivity increases environmental predictability, we might expect that domesticated male zebra finches would have elevated testosterone relative to wild male zebra finches. Whereas wild zebra finches are nomadic (Zann, 1996) and likely travel through high- and low-quality habitats, domesticated zebra finches have experienced generations of high-quality conditions for breeding. This prolonged change in environmental conditions could select for further elevations in circulating testosterone of domesticated males. Indeed our survey of the literature suggests that circulating testosterone levels may be elevated in domesticated male zebra finches across reproductive states (including non-breeding condition) (Table 3). Interestingly, our data also show that domesticated female zebra finches may have elevated plasma steroid levels, suggesting there may have been selection for elevated steroids in domesticated female zebra finches as well as males.

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4.3. Additional potential constraints on circulating sex steroid levels in wild zebra finches

While unpredictable environmental conditions may be one constraint on circulating steroid levels in wild zebra finches, there are other potential constraints. Elevated circulating testosterone has many "costs" (Wingfield et al., 2001), including (1) suppression of the immune system (Folstad and Karter, 1992; Roberts et al., 2004), (2) reduction of fat stores, and (3) elevation of basal metabolic rate (Buchanan et al., 2001).

Testosterone can also have costs for monogamous pair bonding. Monogamous avian species generally have lower circulating testosterone levels than polygynous avian species (Garamszegi et al., 2005). In fact, elevated circulating testosterone in males is associated with an increase in extra-pair paternity across species (Garamszegi et al., 2005). Further, testosterone administration increases extra-pair copulations in sparrows (Wingfield, 1984). While domesticated zebra finches are highly sexually monogamous compared to many songbirds, they do have much higher levels of extra-pair paternity than wild zebra finches (Griffith et al., 2010). This relationship raises the possibility that selection for a different mating strategy in domesticated zebra finches could have had a concomitant influence on circulating testosterone levels.

Decreased female mate choice in domesticated zebra finches could result in an increase in extra-pair courtship in males, if females choose mates based on their perceived commitment to the partnership. Indeed, females appear "less choosy" during mate choice tests in some domesticated zebra finches (Rutstein et al., 2007). Alternatively, in domesticated populations, there may be greater selection for testosterone-dependent mating behaviors by males that secure additional mating opportunities. Forstmeier et al. (2011) found higher levels of extra-pair paternity and male competition in domesticated zebra finches, relative to wild-caught zebra finches. Taken together, these results raise interesting questions about the effect of domestication on mating systems and social behavior in zebra finches.

4.4. Conclusions

Overall, the present results from LC–MS/MS analyses suggest that domesticated zebra finches generally have higher levels of circulating steroids, including testosterone and other androgens and progestins, than wild zebra finches. Increased environmental predictability or other factors during domestication might have selected for higher circulating androgen levels in domesticated zebra finches. These results highlight the importance of considering the potential effects of strain and reproductive state in research on domesticated zebra finches, and suggest that extrapolations from domesticated to wild birds (or vice versa) need to be qualified and tested. Furthermore, these results raise interesting questions about the effect of domestication on mating systems and social behavior in zebra finches.

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