

Endocrine correlates of alternative phenotypes in the white-throated sparrow (*Zonotrichia albicollis*)

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Abstract

Many vertebrate species exhibit alternative phenotypes (or morphs), in which one sex displays phenotypic variation equal to or greater than the variation between the sexes. Males in such species typically display differences in reproductive strategies and morphology. Steroid hormones such as testosterone are known modulators of reproductive behavior and morphology and therefore are obvious candidates for the mediation of phenotypic differences between morphs. We conducted a year-round study in the white-throated sparrow (*Zonotrichia albicollis*) that exhibits alternative phenotypes in plumage coloration and behavior in both sexes: during the breeding season, white-striped males and females are more aggressive and have higher song rates than tan-striped individuals. At the beginning of the breeding season, free-living white-striped males had higher plasma testosterone concentrations than tan-striped males. However, this finding might have been due to different social experiences because captive male morphs sampled at similar times of year did not differ in testosterone concentrations. Captive white-striped males had larger testis and cloacal protuberance sizes than tan-striped males, which might be related to the divergent mating strategies of the morphs. Male morphs showed similar increases in luteinizing hormone following injections of gonadotropin-releasing hormone, but white-striped males showed larger increases in testosterone, indicating differences between morphs in gonadal testosterone production. Females had low concentrations of testosterone, and morphs did not differ. Plasma dehydroepiandrosterone (DHEA) concentrations were elevated in both sexes and morphs during the breeding and non-breeding seasons. These data do not support the hypothesis that testosterone activates behavioral differences between alternative phenotypes in the white-throated sparrow. Alternative testable hypotheses include hormonal effects during early development and direct genetic effects.

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Introduction

Alternative phenotypes, in which individuals of one sex demonstrate dramatic variation in behavioral and/or morphological phenotypes, are present in numerous vertebrate species (Moore et al., 1998; Sinervo et al., 2000; Oliveira et al., 2001; Rhen and Crews, 2002). Such within-sex variation occurs most frequently in males and is often manifested as differences in reproductive behavior and morphology (Taborsky, 1994; Gross, 1996; Knapp, 2004). It is thought that alternative phenotypes evolutionarily persist in a population if each phenotype has a

context-dependent fitness advantage over other phenotypes (Sinervo and Zamudio, 2001; Rhen and Crews, 2002; Shuster and Wade, 2003). In many species with alternative phenotypes, variation within one sex can be equal to or even exceed the variation displayed between the sexes. How is such behavioral and morphological variation generated?

Hormones are key regulators of morphology and behavior and thus are obvious candidates for the mediation of alternative phenotypes (Moore, 1991). In particular, the steroid hormone testosterone has been demonstrated to regulate reproductive and aggressive behavior in male vertebrates during the breeding season (Svare, 1983; Barfield, 1984; Archer, 1988; Wingfield and Farner, 1993; Wingfield and Silverin, 2002). Testosterone and related steroids have therefore been the focus of studies on

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the endocrine mediation of alternative phenotypes. Indeed, in many species of lower vertebrates in which males display alternative phenotypes, the more territorial and aggressive morph has higher concentrations of potent androgens compared to the non-territorial satellite or sneaker morph (summaries in Godwin and Crews, 2002; Knapp, 2004; Oliveira, 2004; but see Moore et al., 1998). Furthermore, experimental manipulation of androgen concentrations in some of the fish and reptile species studied altered the behavioral phenotype of males (Sinervo et al., 2000; Oliveira et al., 2001; Wikelski et al., 2005; but see Weiss and Moore, 2004; Lee and Bass, 2005), suggesting that androgens could be involved in mediating the behavioral phenotypes in lower vertebrates.

Males of several bird species also express alternative phenotypes, but the underlying endocrine mechanisms have remained elusive. In the ruff (*Philomachus pugnax*), male phenotypes differ in territorial behavior and plumage, and the expression of the phenotype likely is autosomally inherited (Lank et al., 1995). Androgens activate the seasonal expression of this trait but do not seem to cause behavioral differences directly (Lank et al., 1999; Rhen and Crews, 2002). The only other avian species expressing alternative phenotypes for which there is extensive behavioral and some endocrinological data is the white-throated sparrow (*Zonotrichia albicollis*). These birds occur in two phenotypes that differ in plumage coloration and behavior: white-striped birds have a white crown stripe, are more aggressive and sing more than birds with a tan crown stripe (Lowther, 1961; Ficken et al., 1978; Watt et al., 1984). Unusual among vertebrate species with alternative phenotypes, the two morphological and behavioral phenotypes are expressed in both sexes (Lowther, 1961). White-striped females are as aggressive as tan-striped males and will spontaneously sing and defend territories in the absence of her mate, while the tan-striped female does not sing nor defend her territory (Lowther, 1961; Kopachena and Falls, 1993; Tuttle, 1993; Collins and Houtman, 1999). Like in the ruff, the expression of alternative phenotypes in the white-throated sparrow has an autosomal genetic basis: the expression of the white-striped morph is perfectly correlated with a pericentric inversion on the second chromosome (Thornycroft, 1975), while tan-striped birds have non-inverted chromosomes and presumably represent the ancestral phenotype (Rising and Shields, 1980). Behavioral differences between the morphs of the white-throated sparrow are fixed and most pronounced during the breeding season (Ficken et al., 1978; Falls and Kopachena, 1994; Collins and Houtman, 1999).

Several prior studies in the white-throated sparrow did not detect differences in circulating testosterone concentrations between the morphs, either in free-living birds during the breeding season (DeVoogd et al., 1995) or in free-living and captive birds during the non-breeding season (Schlinger, 1987; Schwabl et al., 1988). However, two recent studies did report neuroendocrine differences between the two morphs: during the breeding season, white-striped males had higher circulating testosterone concentrations (Swett and Breuner, 2003) and higher arginine vasotocin immunoreactivity in various brain areas compared to tan-striped males (Maney et al., 2005).

Some of the divergence in these findings on endocrine differences between white-throated sparrow morphs could be a result of studies being conducted at different times of year. Furthermore, the social environment could have differed between studies, which is known to have strong influence on circulating hormone concentrations (e.g., Wingfield et al., 1990). To help resolve the question whether the morphs of the white-throated sparrow differ in endocrine organization, we obtained blood samples across seasons in free-living individuals. Furthermore, we took advantage of the rare opportunity to examine endocrine mechanisms underlying alternative phenotypes in both sexes by sampling both male and female morphs. From these samples, we analyzed the concentrations of the steroid hormones testosterone, 17 β -estradiol and dehydroepiandrosterone (DHEA). To test whether the social environment affects hormone concentrations in free-living birds, we sampled a population of captive birds in outdoor aviaries under natural conditions at similar seasonal times and analyzed concentrations of the same sex steroids as well as luteinizing hormone (LH). Finally, to test whether morphs differed in their capacity of gonadal steroid production, we injected captive birds at the beginning of the breeding season with a standard amount of gonadotropin-releasing hormone (GnRH) and compared resulting increases in plasma LH and testosterone in morphs of both sexes.

Materials and methods

All protocols used have been approved by the Princeton University Institutional Animal Care and Use Committee (IACUC Permit # 1516) and are in accordance with the NIH *Guidelines for the Care and Use of Laboratory Animals*.

Bird capture

Birds were captured in Japanese mist nets and baited potter traps at two field sites: during the breeding season (2003–2004) on Stratton Mountain in Stratton, Vermont (43°5'N, 72°56'W) and during the non-breeding season (2002–2005) at the Stony Ford Biological Station in Princeton, NJ (40°4'N, 74°7'W). Upon capture, a blood sample and morphological measurements (tarsus length, wing chord length and furcular fat stores) were taken. Birds were also weighed to the nearest gram, banded with a numbered United States Fish and Wildlife stainless steel band and a unique combination of color bands for individual identification. Birds were brought into captivity under NJ collection permit #SC 23032 and VT #10 VSA section 4152. Head coloration was scored to determine the morph of each individual using a color brightness scale designed specifically for identifying morphs in white-throated sparrows (Piper and Wiley, 1989). Sex was determined using a combination of morphological measurements (presence or absence of brood patch, size of cloacal protuberance, wing chord length, mass; Schlinger and Adler, 1990) as well as laparotomy and behavioral observations.

Gonad volumes were determined by performing laparotomies whereby birds were anesthetized using Isoflurane (AErrane® Baxter Healthcare Co., IL). A small incision was made between the last two ribs, the skin was retracted and the size of the exposed left gonad was measured. Gonad volume was calculated using the formula $V = 4/3\pi a^2b$; where a is half its width and b is half its length (long axis). The incision was then closed using a tissue adhesive (Vetbond™3M Adhesive), and antibiotic powder was applied to the wound. Birds were then allowed to recuperate in an opaque cloth bag. No birds were lost to either anesthesia or laparotomy. Cloacal protuberance height was measured to the nearest millimeter from the base to the top using calipers.

Bird housing

Captive white-throated sparrows ($N=75$) were housed in a total of twelve outdoor aviaries (3 m long \times 2 m wide \times 2 m tall) at Princeton University. Each aviary housed 6–8 birds in mixed-morph and mixed-sex groups. Birds were fed Kaytee Supreme seed and were given fresh water ad libitum. In addition, the birds received every other day a mixture of mashed cooked chicken eggs sprinkled with vitamins (Golden West Bird Products Daily Supplement III) and dried insect larvae, as well as thawed berries. During molt, birds also received molt mix supplements (Petamine™ Molting Formula).

Blood collection

Free-living and captive birds were bled at monthly intervals. Blood samples were collected as fast as possible after capture and maximally within 30 min of capture in both wild and captive birds. During the breeding season, the average time from capture until blood sampling for males was 19.4 ± 0.79 min (mean \pm SE, $n=104$). In general, it took longer to obtain a blood sample in field males (23.51 ± 1.37 min, $n=32$) than in captive males (17.6 ± 0.9 , $n=72$). A univariate ANOVA showed no differences in the time to obtain a blood sample between morphs ($F_{(1,90)}=1.13$, $p>0.29$), but a difference between captive and free-living birds ($F_{(1,90)}=11.1$, $p=0.001$), month ($F_{(3,90)}=6.82$, $p<0.0005$) and an interaction between free-living/captivity and month ($F_{(2,90)}=7.62$, $p=0.001$). Post hoc t tests showed that there was no difference in the times to obtain a blood sample in May (the month in which we detected differences between the morphs in the field but not in captivity, see in Results; $t=-1.5$, $p>0.15$), nor in July ($t=1.9$, $p>0.09$). In June, it took longer to obtain a blood sample from field birds compared to captive birds ($t=4.38$, $p<0.0005$). It is important to analyze the time to take a blood sample because the stress of capture might reduce testosterone levels and increasingly so after longer periods of handling (Wingfield, 1988; Moore, 1991; Sapolsky et al., 2000). However, the time to obtain a blood sample did not affect testosterone concentrations in males during the breeding season (univariate ANOVA, $F_{(1,89)}=0.25$, $p>0.62$). Furthermore, bivariate correlations showed no significant correlation of time to obtain a blood sample and testosterone concentrations for any month (Spearman's correlation, all $p>0.19$) except for June, when there was a positive (but probably spurious) correlation (Spearman's $\rho=0.44$, $p=0.01$).

The time it took to obtain a blood sample in females during the breeding season was similar to that of males (17.92 ± 1.03 min), and there were no differences between free-living and captive females, morphs or months (univariate ANOVA, all effects $p>0.27$).

Samples (~ 300 μ l) were collected by puncturing the brachial vein with a 26-gauge needle and drawing up the blood in (50 μ l) micro-hematocrit capillary tubes. Plasma was separated from the red blood cells by centrifugation using a standard field centrifuge at $500 \times g$ for 5 min (Hau et al., 2004a) and stored at -20°C until hormone analysis (Wingfield and Farner, 1975). We made an effort to avoid repeated bleeding of individuals within a year. Out of a total of 92 free-living individuals that were bled in the field over the course of the study, only four white males were bled repeatedly in 2003 and 2004 (all during the breeding season). Individuals from the captive population were bled repeatedly within a year and between years but for various logistical reasons we were not able to obtain samples from the same set of individuals during each sampling period. We therefore randomized individuals to be bled each month in an attempt to control for individual effects.

Hormone analyses

Sex steroids

Samples were assayed for testosterone and 17β -estradiol (E_2) content by radioimmunoassay (RIA) after partial purification on diatomaceous earth/glycol columns (Wingfield and Farner, 1975; Ball and Wingfield, 1987; Hau et al., 2004b). Control samples containing only water were taken through each entire assay and were always below the lower threshold of detectability. Twenty microliters of tritiated steroid were added to each sample to determine the percentage recovery after extraction of samples with dichloromethane. Antibodies were purchased from Research Diagnostics Inc., Flanders, NJ (testosterone) and from Biogenesis, Poole, England (E_2).

Samples from free-living and captive individuals and from different seasons and sexes were randomly assigned to each assay. Testosterone samples were analyzed in a total of 11 assays. Average lower detection limit for testosterone was 0.091 ± 0.009 ng/ml, average recovery was $53.8 \pm 3.78\%$. Average intra-assay variation for testosterone was 16.24%, and inter-assay variation was 23.2%. We measured E_2 in a subset of individuals in a total of 4 assays with an average lower detection limit of 0.202 ± 0.103 ng/ml and an average recovery of $50.75 \pm 8.3\%$. However, 97% of the samples (74 out of 76) had E_2 concentrations below the lower detection limit of our assay, therefore samples and data were not analyzed further.

Dehydroepiandrosterone (DHEA)

Samples were measured in a total of eight direct RIAs (with an average plasma volume of 40 μ l and analyzed without prior column chromatography following the protocol in Hau et al., 2004b). The DHEA antibody was purchased from Endocrine Sciences, Tarzana, CA. Average lower detection limit was 0.27 ± 0.06 ng/ml, and average recoveries were $77.12 \pm 2.6\%$. Average intra-assay variation was 18.6%, and inter-assay variation was 12.2%.

Luteinizing hormone (LH)

LH was measured in two assays in the Wingfield Lab at the University of Washington using a double-antibody post-precipitation radioimmunoassay (Follett et al., 1972, 1975). The intra-assay variation was 4.3%, and inter-assay variation was 9.1%.

GnRH injections

At the beginning of the breeding season in May, we measured the effect of GnRH injections on plasma LH and androgens concentrations in captive male and female white-throated sparrows. Birds were injected with a solution of 500 ng/10 μ l chicken-1 luteinizing hormone releasing hormone (Sigma-Aldrich Corp.) dissolved in 0.8% saline (Sigma-Aldrich Corp.) into the jugular vein using a 25- μ l Hamilton syringe. This dose has been found to cause a maximal LH response in closely related male and female song sparrows (*Melospiza melodia*; Wingfield and Farner, 1993). Control birds received a saline injection. A blood sample for baseline testosterone was taken immediately prior to injection, and then birds were bled again at 5 min to measure LH and at 30 min to measure their gonadal androgen response to injection. Birds were randomly assigned to either GnRH or saline injection for the first round of injections (Week 1, May 10–14, 2004). Each bird then served as its own control in a repeated-measures design and received the opposite treatment 2 weeks later (Week 2, May 23–28). LH samples for this experiment were run in a single assay. Testosterone concentrations were analyzed using column chromatography in a total of 3 assays. Average recoveries for the testosterone assays were 54.17 ± 12.1 , and the lower detection limit was 0.083 ± 0.03 . Intra-assay variation for the testosterone assays was 13.7%, and inter-assay variation was 44%. Due to issues with column chromatography, we obtained high inter-assay variation, however, white-striped and tan-striped birds were randomly assigned to assays. Indeed, white-striped males that responded strongly to GnRH injection with testosterone production were represented in each assay, which renders us confident that assay variation did not obscure morph differences. There was no effect of injection order (saline versus GnRH injection) on LH or androgen response.

Statistical analysis

All data are reported as means ± 1 standard error of the mean and were analyzed using SPSS 12.0 for Windows (SPSS Inc., Chicago). Data were transformed to meet assumptions for parametric statistics when necessary (see Results). Since almost all free-living birds were sampled only once and captive birds were sampled as randomly as possible, we used statistics for independent data to analyze present data. Because of known large endocrine differences between the sexes, all statistical tests were conducted separately for each sex. t tests were used to compare morphological measurements between morphs. Because of the expected large increases in circulating hormones during the

breeding season, we first lumped months into either breeding season (April through July) or non-breeding season (August through March) and used *t* tests to analyze seasonal changes in hormone concentrations in both morphs combined. We then tested for differences between morphs and time (months) within the breeding season using univariate ANOVAs followed by post hoc *t* tests. Because of a recent discussion of the validity of Bonferroni corrections (Moran, 2003), here, we report the *p* value for the post hoc *t* test side-by-side with the value for the Bonferroni-corrected α . When sample sizes were small (for LH and female hormones), we lumped data into breeding and non-breeding season and used univariate ANOVA to test for season and morph differences. Effects of captivity on testosterone were analyzed using *t* tests only during the breeding season because testosterone were very low during the non-breeding season.

In the GnRH experiment, LH concentrations were compared using a repeated-measures ANOVA with treatment (saline or GnRH injection) and morph as factors. Because of limited sample sizes, we first analyzed the effect of injection type (saline versus GnRH) on androgen concentrations before injection versus 30 min after injection using a *t* test (morphs lumped). We then computed the testosterone response to GnRH injection in each individual by subtracting testosterone concentrations 30 min after GnRH injection from those before the injection and used a *t* test to analyze morph differences.

Results

Testis and cloacal protuberance sizes

In May, after 1 month in captivity, there were no differences in body mass between the male morphs (*t* test, $t = -1.05$, $p > 0.3$, white males: $n = 22$, 27.7 ± 0.68 , tan males: $n = 7$, 29.2 ± 1.46). However, white-striped females were heavier than tan-striped females (white-striped females: $n = 8$, 24.2 ± 0.74 , tan-striped females: $n = 4$, 20.8 ± 1.39 , *t* test: $t = 2.41$, $p = 0.036$). Testis volume of white-striped males was significantly larger than that of tan-striped males (*t* test, $t = 2.39$, $p = 0.024$, Table 1). The cloacal protuberance of white-striped males was also larger than in tan-striped males (*t* test, $t = 2.4$, $p = 0.024$; Table 1).

Plasma testosterone in free-living and captive birds

As expected, free-living male white-throated sparrows had higher circulating testosterone concentrations during the breeding season compared to the non-breeding season (morphs lumped, breeding $n = 69$, non-breeding $n = 36$, *t* test, $t = 6.99$, $p < 0.0005$; see Fig. 1a). Within the breeding season, testosterone concentrations varied between months (ANOVA, effect of month: $F_{(2,63)} = 4.34$, $p = 0.017$) and also between morphs (interaction month \times morph: $F_{(2,63)} = 4.23$, $p = 0.019$; effect of morph: $F_{(1,63)} = 3.73$, $p = 0.058$, see Fig. 1a). Post hoc *t* tests revealed that white-striped males had higher testosterone concentrations at the beginning of the breeding season in May ($t = 2.87$, $p = 0.021$; Bonferroni-corrected $\alpha = 0.017$), but not in

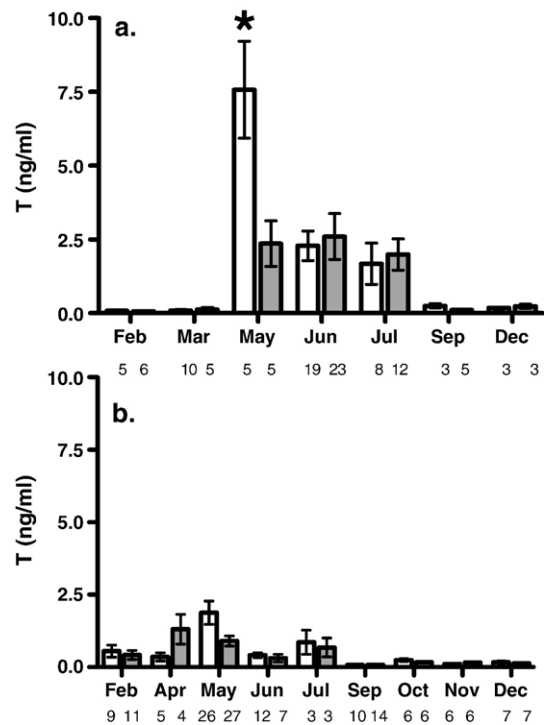


Fig. 1. Circulating plasma testosterone (mean \pm SE) of free-living (a) and captive (b) male white-throated sparrows across the year. White bars indicate white-striped morphs, gray bars indicate tan-striped morphs. Sample sizes for each morph are given below x axis. In both free-living and captive birds, testosterone was higher during the breeding season (Apr–Jul) than during the non-breeding season (Aug–Mar). In May, free-living white-striped males had higher testosterone concentrations than tan-striped males.

the middle or end of the reproductive period ($p > 0.7$ for both Jun and Jul; Fig. 1a).

During the breeding season, circulating testosterone concentrations in captive males were reduced to about half of that of free-living males (morphs lumped: field $n = 69$, captive $n = 78$; $t = 3.96$, $p < 0.0005$). Captive males had higher circulating testosterone concentrations during the breeding compared to the non-breeding season (morphs lumped, breeding $n = 78$, non-breeding $n = 90$; *t* test, $t = 4.85$, $p < 0.0005$, Fig. 1b). However, in captive males during the breeding season, there was no difference in plasma testosterone either between months or morphs (ANOVA, all effects $p > 0.6$).

Circulating testosterone concentrations were generally much lower in female than in male white-throated sparrows. In general, females had higher circulating testosterone concentrations during the breeding season compared to the non-breeding season, but seasonal differences were much smaller than in males (morphs lumped, *t* test on breeding versus non-breeding season: free-living females: $t = 0.451$, $p > 0.6$; captive females: $t = 3.27$, $p = 0.002$; Table 2). Within the breeding season, there were no differences between the morphs in either free-living ($t = -1.13$, $p > 0.2$) or captive females ($t = 1.77$, $p = 0.08$). Perhaps due to overall low testosterone concentrations, there were no differences in testosterone between free-living and captive females (morphs lumped: breeding $n = 23$, non-breeding $n = 48$, $t = 1.53$, $p > 0.1$).

Table 1

Testis volumes and cloacal protuberance (CP) lengths (mean \pm SE) of male white-striped and tan-striped white-throated sparrows at the beginning of the breeding season

	White-striped	Tan-striped
Testis volume (mm ³)	13.07 \pm 2.12* (19)	7.01 \pm 1.36 (10)
CP length (mm)	4.34 \pm 0.212* (17)	3.74 \pm 0.132 (9)

Numbers in bars indicate sample sizes. *White-striped males had larger testis volumes and CP lengths.

Table 2
Plasma testosterone concentrations (ng/ml; mean±SE) in free-living and captive female white-throated sparrows

Season	Free-living		Captive	
	White-striped	Tan-striped	White-striped	Tan-striped
Breeding	0.23±0.04 (14)	0.43±0.2 (8)	0.21±0.03 (26)	0.16±0.08 (19)
Non-breeding	0.2±0.07 (7)	0.55±0.46 (10)	0.07±0.01 (12)	0.14±0.02 (16)

Plasma DHEA in free-living and captive birds

Free-living males had elevated concentrations of DHEA in both the breeding and the non-breeding season. However, there were no seasonal (breeding vs. non-breeding) or morph differences in male DHEA (months lumped, ANOVA, reciprocal transformed, all effects $p>0.1$; Fig. 2a). During the pre-breeding season (February and March), we noticed that white-striped males had significantly higher circulating DHEA concentrations ($n=7$, 2.51 ± 0.34 ng/ml) than tan-striped males ($n=9$, 1.19 ± 0.4 ng/ml, t test, $t=2.38$, $p=0.032$). We found no differences between seasons or morphs in captive males (ANOVA, all effects, $p>0.3$, Figs. 2b, d). DHEA of captive males and females did not differ from that of free-living birds either during the breeding or the non-breeding season (ANOVA, morphs lumped, males: reciprocal transformed, all effects, $p>0.6$; females: all effects, $p>0.3$).

Free-living females did not vary in circulating DHEA concentrations between the breeding and the non-breeding

season (ANOVA, reciprocal transformed, effect of season: $p>0.1$) or between morphs (effect of morph, $p>0.5$, Fig. 2c). However, there was a significant season×morph interaction ($F_{(1,27)}=5.34$, $p=0.029$; Fig. 2c) with white-striped females having higher DHEA during the non-breeding season than tan-striped females. Similar to males, free-living white-striped females during the pre-breeding season in February and March appeared to have higher DHEA concentrations than tan females (white-striped females: $n=3$, 2.87 ± 0.3 ng/ml; tan-striped females: $n=2$, 1.13 ± 0.18 ng/ml). In captivity, DHEA also did not differ seasonally (effect of season, $p>0.9$), but white-striped females had generally higher circulating DHEA than tan-striped females (effect of morph; $F_{(1,83)}=5.78$, $p=0.018$, interaction season×morph, $p>0.8$; Fig. 2d).

Plasma LH in captive birds

Due to the difficulty in obtaining large plasma samples for the analysis of multiple hormones in small birds like white-throated sparrows, concentrations of plasma LH were only measured in captive but not in free-living birds (Table 3). As expected, males had significantly higher LH concentrations during the breeding compared to the non-breeding season (ANOVA, morphs lumped, reciprocal transformed; effect of season, $F_{(1,93)}=6.53$, $p<0.02$), but there were no differences between the morphs and no interaction between season×morph (all $p>0.4$; see Table 3).

In females, there was no seasonal difference in plasma LH (ANOVA, morphs lumped, reciprocal transformed; effect of

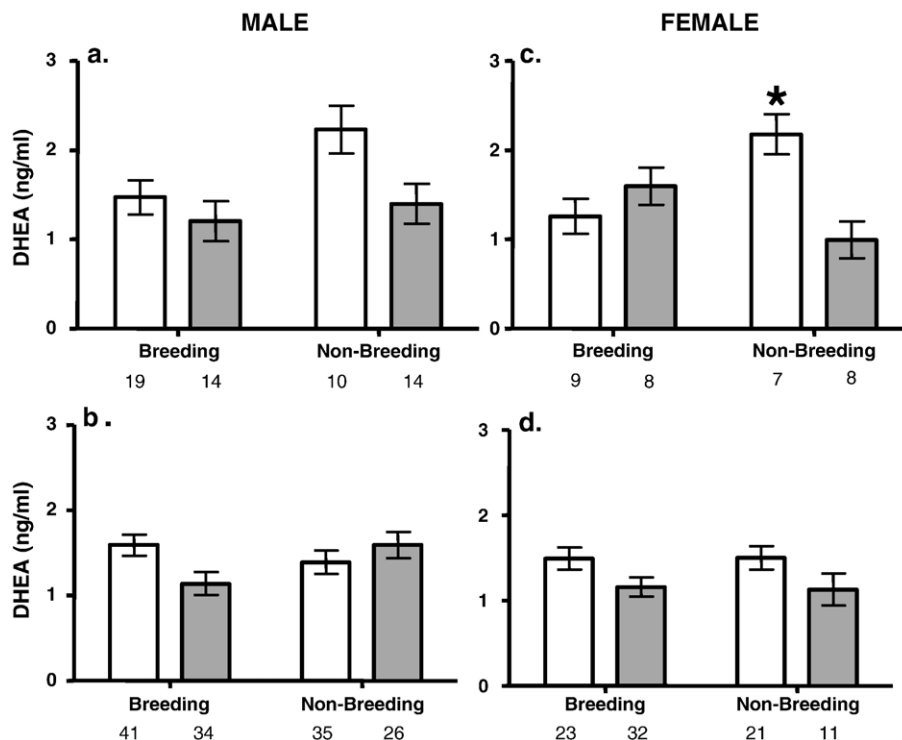


Fig. 2. Plasma DHEA concentrations (mean±SE) of free-living (upper graphs) and captive (lower graphs) male (a, b) and female (c, d) white-throated sparrows in the breeding (Apr–Jul) and non-breeding season (Aug–Mar). White bars indicate white-striped morphs and gray bars indicate tan-striped morphs. Sample sizes are given below bars. DHEA was detectable year-round in both free-living and captive males and females.

Table 3
Seasonal profile of plasma LH concentrations (ng/ml; mean±SE) in male and female white-throated sparrows

Season	Males		Females	
	White-striped	Tan-striped	White-striped	Tan-striped
Breeding	1.35±0.198 (21)	1.46±0.22 (23)	0.728±0.08 (13)	2.15±0.58 (11)
Non-breeding	0.777±0.48 (26)	0.866±0.528 (27)	0.677±0.231 (19)	0.910±0.283 (6)

Sample sizes are given in parentheses. LH was higher during the breeding than during the non-breeding season. There were no morph differences among males, but tan-striped females had higher LH than white-striped females.

season: $F_{(1,45)}=3.69$, $p=0.061$), but tan females appeared to have higher LH than white females (effect of morph: $F_{(1,45)}=19.18$, $p<0.01$; interaction season × morph $p>0.18$, Table 3).

GnRH injections in captive birds

As expected, 5 min after GnRH injection, males had significantly higher plasma LH concentrations than after saline injections (repeated-measures ANOVA, effect of injection, $F_{(1,16)}=19.73$, $p<0.0005$, Fig. 3a). However, there was no difference in plasma LH between male morphs (effect of morph, and interaction injection × morph, all $p>0.1$). Males had higher plasma testosterone 30 min after injection with GnRH than after

Table 4
Plasma testosterone concentrations (ng/ml; mean±SE) of male and female white-throated sparrows before and 30 min after an injection with either saline or GnRH

GnRH	Males		Females	
	White-striped	Tan-striped	White-striped	Tan-striped
Before	(see Fig. 2)		0.16±0.09 (5)	0.17±0.05 (6)
After injection			0.82±0.48 (5)	0.33±0.52 (6)

Saline	Males		Females	
	White-striped	Tan-striped	White-striped	Tan-striped
Before	1.04±0.49 (4)	1.17±0.46 (8)	1.11±0.99 (4)	0.17±0.09 (3)
After injection	0.64±0.17 (6)	1.66±1.06 (6)	0.12±0.04 (5)	0.26±0.11 (3)

injection with saline (paired t test, morphs lumped: $t=3.32$, $p<0.01$, for GnRH-injected birds, see Fig. 3b). To compare the response to GnRH injection between the two morphs, we computed for each individual the difference in plasma testosterone before and 30 min after injection. White-striped males had a stronger response in plasma testosterone than tan-striped males (t test, log-transformed data, $t=2.2$, $p=0.048$).

Like males, females had higher plasma LH after GnRH versus saline injection ($F_{(1,13)}=49.78$, $p<0.0005$, Fig. 3c), however, as in males, there was no difference between the

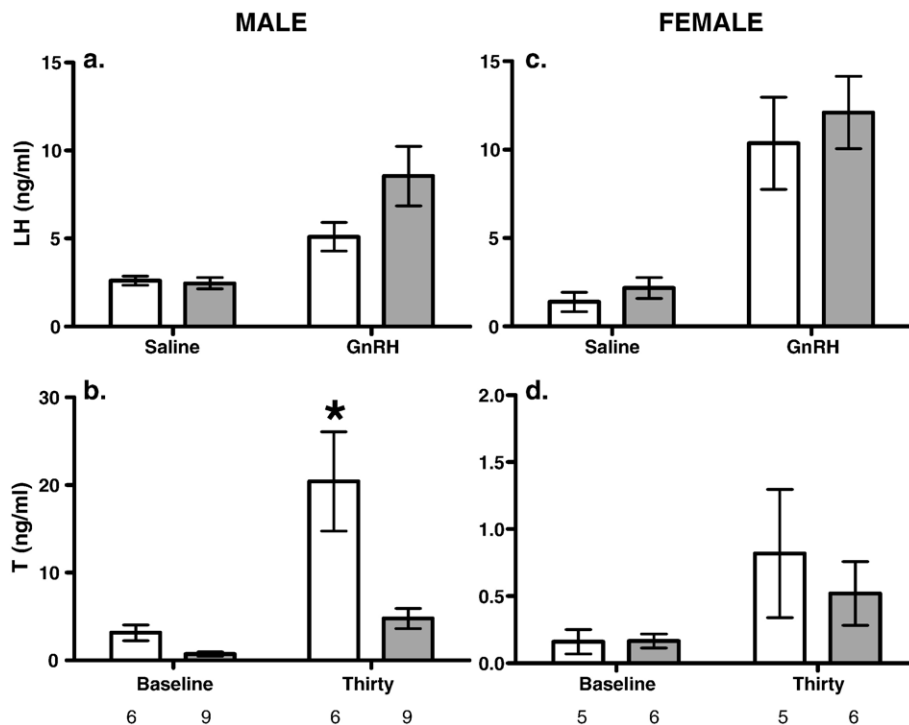


Fig. 3. Plasma T and LH concentrations (mean±SE) in response to GnRH injections in captive male and female white-throated sparrows. White bars indicate white-striped morphs, gray bars indicate tan-striped morph. Sample sizes are given below x axis. (a) Male LH concentrations 5 min after saline or GnRH injection. There was a significant increase in LH after GnRH injection compared to saline injection, but no morph difference. (b) Male plasma testosterone immediately before (baseline) and 30 min after GnRH injection. Males increased testosterone in response to GnRH injection, and white-striped males showed a larger increase in testosterone than tan-striped males. (c) Female LH concentrations 5 min after saline or GnRH injection. Females significantly increased LH after GnRH injection, but not after saline injection. There were no differences between morphs. (d) Female plasma testosterone immediately before (baseline) and 30 min after GnRH injection. There was no significant effect of GnRH injection on testosterone concentrations.

morphs (effect of morph, and interaction between injection \times morph, all $p > 0.3$). Females did not significantly increase testosterone levels 30 min after GnRH injection compared to saline injection (T: $t = 1.25$, $p > 0.2$, Fig. 3d). Female response to GnRH injection (the difference in plasma testosterone before and 30 min after GnRH injection) did not differ between morphs ($p > 0.5$) (Table 4).

Discussion

Do hormones mediate behavioral alternative phenotypes in males?

Like in many species of vertebrates, white-throated sparrows showed seasonal differences in circulating plasma concentrations of testosterone (Wingfield and Farner, 1993). The finding of elevated testosterone concentrations in males during the breeding season renders sex steroids likely candidates for the mediation of behavioral phenotypic differences. Indeed, free-living white-striped males had higher circulating testosterone concentrations than tan-striped males at the beginning of the breeding season (Fig. 1a). These data could suggest that behavioral differences between male morphs during the breeding season are activated by testosterone. However, in captivity, male morphs did not differ in circulating testosterone concentrations (Fig. 1b). Taken together, these data prompt the hypothesis that hormone differences observed in the field during the breeding season are not caused by inherent differences between morphs in testosterone concentrations but that they are the result of differential social or environmental experiences. This hypothesis will be discussed in more detail further below.

Plasma concentrations of 17β -estradiol (E_2) were mostly undetectable in the subset of free-living and captive white-throated sparrows that we tested in the current study. Testosterone can be involved in the control of aggressive behavior (and song) via two pathways: by directly binding to androgen receptors, or after being converted into E_2 by the enzyme aromatase and subsequently binding to estrogen receptors (Callard, 1984; Hutchison and Steimer, 1984). Previous findings detected about 0.4 ng/ml E_2 in captive female white-throated sparrows at the beginning of the breeding season (Archawaranon, 1987), and experiments on during the non-breeding season have suggested that both androgenic and estrogenic pathways are responsible for an activation of aggressive behavior and song (Archawaranon and Wiley, 1988). The lack of detectable E_2 in our study could indicate that estrogen production and action are occurring primarily in the brain (Tsutsui and Schlinger, 2002).

Does social experience cause differences in testosterone between male morphs?

What caused white-striped males to have higher testosterone concentrations than tan-striped males at the beginning of the breeding season? A recent study has suggested that white-striped males settle in areas of higher population density than

tan-striped males (Formica et al., 2004). The reason for this differential settlement pattern is probably connected to the different mating strategies of the two morphs: white-striped males are more polygynous and seek more extra-pair copulations than tan-striped males (Tuttle, 2003). Having more neighbors may increase the access of white-striped males to females and thus their likelihood of obtaining extra-pair copulations (Rising and Shields, 1980; Formica et al., 2004). Thus, the frequency and/or intensity of sexual and aggressive encounters (for example encounters with males at territory boundaries) might be higher in white-striped than tan-striped males. This social stimulation could be responsible for increases in circulating testosterone concentrations in white-striped males (Wingfield et al., 1990), especially at the beginning of the breeding season when social interactions are likely to be highest. Variations in social experience between different study populations could also have contributed to the divergent findings of morph differences in testosterone in the literature (DeVoogd et al., 1995; Swett and Breuner, 2003). We are currently testing the endocrine responses of free-living male white-throated sparrows to a standardized aggressive stimulus, a simulated territorial intrusion (Wingfield, 1985).

In contrast to free-living birds, our captive birds were kept in long-term mixed-morph and mixed-sex groups and probably were living in stable social situations with established dominance hierarchies (Schlinger, 1987; Archawaranon et al., 1991). In such socially stable groups, birds are not expected to show differences in testosterone concentrations (Wingfield and Ramenofsky, 1985; Wingfield et al., 1990). Absolute concentrations of testosterone in captive male white-throated sparrows were greatly reduced compared to free-living conspecifics (Fig. 1), which is a well-known effect either caused by the stress of captivity and a resulting increase in corticosterone secretion which suppresses testosterone or by a reduction of social and sexual interactions (Wingfield, 1988; Moore, 1991; Sapolsky et al., 2000).

Do male morphs differ in gonadal testosterone production?

To test whether the differences between free-living male morphs in circulating plasma testosterone are due to different social (or other environmental) experiences or whether the morphs differed in their capability to produce testosterone, we injected captive birds with a standardized amount of GnRH. GnRH is a potent and fast-acting stimulator of LH and thereby androgen secretion (Wingfield and Farner, 1993). Interestingly, in response to GnRH injection, white-striped males showed a greater increase in testosterone concentrations than tan-striped males (Fig. 3b). LH responses to GnRH injection, by contrast, were similar among male morphs (Fig. 3a). These data suggest that pituitary responsiveness (LH release) to GnRH is similar in the two morphs but that the testes of white-striped males respond with higher testosterone release. The physiological mechanisms underlying the differential testosterone production are still unclear, but testable hypotheses include a differential sensitivity of the two morphs to the LH signal (perhaps due to variation in the abundance of LH receptors in testosterone-

producing Leydig cells) or in the abundance and activity of androgenic enzymes in Leydig cells resulting in differential testosterone production rates.

Do male morphs differ in reproductive physiology?

At the beginning of the breeding season (after 4 weeks in captivity), male morphs differed in the size of two reproductive organs: white-striped males had larger testis volumes and larger cloacal protuberances than tan-striped males (Table 1). Such differences have not been found in this species previously, either in free-living or captive males (DeVoogd et al., 1995; Maney et al., 2005; though there is a similar trend in DeVoogd et al., 1995). Testis size is a poor predictor of circulating testosterone concentrations (in other *Zonotrichia* spp.: Moore et al., 2002) but is a good indicator of sperm production (Møller, 1988; Stockley et al., 1997). Likewise, the cloacal protuberance functions in sperm storage and delivery (Birkhead et al., 1993; Rising, 1996; but see Sax and Hoi, 1998). Thus, these differences in testis and cloacal protuberance sizes of male morphs could be related to their different mating strategies and the fact that white-striped males seek more extra-pair copulations than do tan-striped males (see above; Tuttle, 2003).

Do hormones mediate behavioral phenotypes in females?

Female white-throated sparrows showed seasonal variations in plasma testosterone, but absolute concentrations of testosterone during the breeding season were about one order of magnitude lower than in males (Table 2). Female morphs did not differ in circulating testosterone concentrations during the breeding season, but small sample sizes, less frequent sampling times and much lower concentrations of testosterone than in males could have obscured morph differences. Female white-throated sparrows did not significantly increase testosterone after GnRH injections, but sample sizes were relatively low. The endocrine control of aggressive behavior and song in female birds has remained unresolved (e.g., Wingfield, 1994; Ketterson et al., 2005). One possible scenario is that in female birds local steroid synthesis and action in the brain might be responsible for regulating in aggressive behavior and song (Schlinger, 1998; Goymann and Wingfield 2004).

Is DHEA involved in controlling behavior?

Recently, the adrenal steroid precursor DHEA has gained attention for its possible involvement in the control of aggressive behavior in vertebrates, as well as in song production in birds (Soma and Wingfield, 2001). It has been suggested that DHEA could be used locally in the brain as a substrate for the synthesis of active sex steroids to regulate behaviors when needed (Soma and Wingfield, 2001). Indeed, plasma DHEA is elevated in song sparrows in spring and winter when they sing and aggressively defend their territories, but is significantly reduced during molt when these behaviors are not expressed (Soma and Wingfield, 2001). Furthermore, administration of DHEA increases singing

in response to territorial intrusions in non-breeding song sparrows (Soma et al., 2002), and plasma concentrations of DHEA are positively correlated with aggressive behavior in tropical spotted antbirds during the non-breeding season (*Hylophylax n. naevioides*; Hau et al., 2004b).

DHEA was present at elevated concentrations in both the breeding and the non-breeding season in free-living and captive white-throated sparrows, and in general there were no season, sex or morph differences (Fig. 2). However, free-living white males and females tended to have higher DHEA concentrations than their tan counterparts during the non-breeding season (Fig. 2), and a more detailed examination of the data suggests that white morphs of both sexes had higher circulating DHEA during the pre-breeding season in Feb/Mar. While still preliminary, these findings suggest that (1) DHEA is available as a steroid precursor year-round and (2) DHEA might be involved in generating behavioral differences between white-striped and tan-striped morphs during the pre-breeding season. Plasma concentrations of DHEA were not reduced in captivity (Fig. 2), suggesting that the production and/or metabolism of this hormone is less susceptible to stress or lack of social stimuli. This result is consistent with studies on song sparrows and zebra finches showing that acute stress did not result in a change in plasma DHEA (Soma and Wingfield, 2001; Soma et al., 2004).

Conclusions

Our data suggest that testosterone activates seasonal reproductive behaviors in male and female white-throated sparrows during the breeding season as in many other species. However, the lack of differences between morphs in circulating sex steroids in captivity suggests that plasma testosterone is not directly involved in generating alternative phenotypes in behavior. The observed differences between male phenotypes in testis and cloacal protuberance sizes might be related to the different mating strategies of the morphs. White-striped males also showed a higher GnRH-induced testosterone production, the physiological and functional reasons of which are still obscure.

If behavioral differences between alternative phenotypes in the white-throated sparrow are not the result of activational endocrine effects, how are they generated? Preliminary data on circulating concentrations of 5α -dihydrotestosterone (DHT) indicated no differences between the morphs in either sex (Spinney and Hau, unpublished data). However, endocrine factors could still be involved but could act to during early development to permanently differentiate behavioral differences in the two morphs like in male tree lizards (*Urosaurus ornatus*; Moore, 1991; Moore et al., 1998). Alternatively, behavioral phenotypes might primarily be generated by direct genetic mechanisms, for example, by differences in brain structures, receptor or enzyme dynamics or downstream processes (Halpern-Sebold et al., 1986; Arnold, 1996; Rhen and Crews, 2002). We are currently testing several of these hypotheses using experimental manipulations of social experience and hormone actions in white-throated sparrows.

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