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Plasma Testosterone Correlates with Morph Type across Breeding Substages in Male White-Throated Sparrows

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ABSTRACT

White-throated sparrows (*Zonotrichia albicollis*) exhibit a genetic polymorphism that affects plumage and behavior in both sexes. White-striped morphs are more territorially aggressive, whereas tan-striped morphs provision nestlings at a higher rate. We investigated testosterone physiology in this species in an effort to understand hormonal mechanisms for the observed differences in aggression and parental care between the morphs. We found a small but significant difference in plasma testosterone between free-living white-striped and tan-striped males over the course of the breeding season. This difference correlates with previously observed differences in aggressive behavior and suggests that testosterone may mediate these differences. Testosterone remained higher in white-striped males relative to tan-striped males when males were provisioning nestlings and fledglings. Thus, testosterone may also contribute to the relatively reduced levels of parental care exhibited by white-striped males. In contrast to males, plasma testosterone did not differ between free-living white-striped and tan-striped females, which suggests that testosterone does not mediate differences in aggression between female morphs. Injection with gonadotropin-releasing hormone led to greater testosterone secretion in both captive and free-living males and captive females but did not differ by morph. Therefore, we conclude that differences in plasma testosterone between the morphs are due to differences in testosterone regulation upstream of the pituitary.

Introduction

Hormone/behavior relationships are inherently noisy, so noisy, in fact, that relationships are best identified between individuals exhibiting extreme values of the behavior. Unfortunately, these individuals are rare in any population, making the comparison

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difficult. One can circumvent this problem through multispecies comparisons, but this approach introduces confounding factors such as genetic background, species ecology, and environment. Another approach that has proved successful is phenotypic engineering (Reed et al. 2006), in which treatment with exogenous hormones is used to exaggerate differences between endocrine phenotypes within the same species.

Behaviorally polymorphic species offer a natural case of phenotypic engineering, providing exaggerated, often bimodal distributions of a behavioral trait within a single population. This allows comparison of individuals that exhibit distinct behavioral phenotypes, making hormone/behavior relationships easier to detect. Thus, these species are a powerful tool for studying the endocrine bases of behavior.

The majority of behaviorally polymorphic species vary in reproductive strategies among males, the most common variations being a territorial, aggressive phenotype and a less aggressive "sneaker" or "satellite" phenotype (Brantley et al. 1993; Lank et al. 1995; Sinervo and Lively 1996). These species have been used very successfully to study the hormonal and neural mechanisms of courtship behaviors and territorial aggression. The white-throated sparrow (*Zonotrichia albicollis*), however, exhibits a very different behavioral polymorphism that is distinguished by two important features. First, morph types may be roughly classified as a territorially "aggressive" morph that expends more effort in pursuit of extrapair matings and a "parental" morph. White-striped (WS) birds are more aggressive in response to simulated territorial intrusion, and this difference persists into the parental phase (Kopachena and Falls 1993*a*). WS birds are also estimated to have higher rates of extrapair copulation (based on rates of intrusion into neighboring territories; Tuttle 2003) and to sing more frequently than tan-striped (TS) birds (Falls and Kopachena 1994). TS birds, the parental morph, provision nestlings at a higher rate than their WS counterparts (Kopachena and Falls 1993*b*). This aggressive/parental distinction represents a dichotomy very different from the more common territorial/sneaker morph types and illustrates what is thought to be a fundamental trade-off between mating effort and parental care (Trivers 1972). Thus, this species presents an ideal opportunity to study hormonal mechanisms of this trade-off. Second, morph type is determined by a pericentric inversion on the second somatic chromosome and is not sex linked (Thorneycroft 1966, 1975). Thus, females also exhibit both morph types. This presents a unique opportunity to study the endocrine bases of aggressive and parental behavior in females as well as males.

Data from many taxa suggest that testosterone (T) may mediate the trade-off between mating effort and parental care (Wingfield et al. 1990; Reburn and Wynne-Edwards 1999; Flem-

ing et al. 2002; Young et al. 2005). In the breeding season, T and other androgens are positively associated with the aggressive and sexual behaviors that are more pronounced in the WS morph (Balthazart 1983; Wingfield et al. 1987; Schwabl and Kriner 1991). In contrast, experimentally elevated T often leads to a reduction in parental care behavior (Schoech et al. 1998; Van Roo 2004; Schwagmeyer et al. 2005), and males that provide parental care usually show reduced T levels when they enter the parental stage of the nesting cycle (Wingfield et al. 1987). However, T does not always lead to a reduction in parental care. In species where breeding seasons are abbreviated or male parental care is essential, paternal behavior may be insensitive to T (Lynn et al. 2002, 2005; Van Duyse et al. 2002).

We measured T levels in white-throated sparrows to determine whether differences in behavior between morphs correspond to differences in T levels. In addition, we monitored individual nests to examine how T levels changed in each morph over the course of the nesting cycle. Given the differences in aggressive and sexual behavior between the morphs, we predicted that WS birds, both male and female, would have higher levels of plasma T than TS birds during the defense phase. We also predicted that this difference in T will persist through the nesting phase, contributing to differences in parental care between morphs. We also performed a gonadotropin-releasing hormone (GnRH) challenge to determine possible mechanisms underlying the proposed difference between morphs.

Material and Methods

Study Species

The white-throated sparrow is a migratory songbird that breeds in the northeastern United States and Canada and winters in the southeastern United States. This species is primarily socially monogamous (Tuttle 2003). Males assist with feeding of nestlings and fledglings but do not incubate (Falls and Kopachena 1994). Sparrows in our study population (Northwoods Stewardship Center in East Charleston, VT: 44°50′13″N, 71°59′24″W) may rear two broods per season and will renest three or more times if nests are depredated (M. B. Swett, personal observation).

Collection of Field Samples

Samples were collected between May 22 and July 29, 2003, between May 2 and July 23, 2004, and between April 21 and July 10, 2005. A total of 53 males (20 TS and 33 WS) and 37 females (13 WS and 24 TS) were sampled. Sparrows were captured in seed-baited Potter traps or mist nets (there was no difference in T levels between trapped and netted birds; t-test: $P = 0.54$). Either mist nets were placed near the nest or birds were attracted to the net with playbacks of conspecific songs. Blood samples were collected by venipuncture of the alar vein, and blood was drawn into a heparinized microhematocrit tube via capillary action. Samples used to measure T were collected within 10 min of the bird contacting the net or the fieldworker

approaching the potter trap (three were collected within 15 min) so as to minimize the effect of stress on T levels (Moore et al. 2000; Lance et al. 2004). All birds were banded with a U.S. Fish and Wildlife Service numbered band as well as a unique combination of colored plastic leg bands, allowing birds to be identified visually at a distance. Nests were located and monitored for as many individuals as possible (46 males and 31 females) in order to determine the stage in the nesting cycle at which each sample was taken.

Blood samples were kept on ice in the field (up to 4 h). Each sample was then centrifuged, and plasma was drawn off with a Hamilton syringe. Plasma was kept frozen at approximately −20°C until it could be assayed.

GnRH Challenge

Lab. Wintering sparrows were captured in seed-baited Potter traps or seed-baited mist nets at the Brackenridge Field Station of the University of Texas and the Center for Environmental Research at Hornsby Bend in Travis County, Texas (30°20′00″N, 97°48′00″W). Birds were housed in individual 13 × 15 × 17-inch cages in captivity and were photostimulated (14L : 10D) for 3 wk to bring them into pseudo–breeding condition before the start of the experiment. A total of 21 males (14 WS and 7 TS) and 10 females (5 WS and 5 TS) were used in this experiment.

We followed a GnRH challenge protocol optimized in dark-eyed juncos, a closely related and similarly sized species (Jawor et al. 2006). A 100-μL blood sample was taken from each individual via venipuncture of the alar vein. Birds were then given either 50 μL GnRH in saline (25 ng/μL) or saline alone injected intramuscularly into the pectoralis. They were then held in a cloth bag for 30 min, at which point a second blood sample was taken. One week later, the experiment was repeated as described, except that individuals that had received the GnRH treatment now received a control injection, and vice versa.

Field. The GnRH challenge experiment was repeated using free-living breeding birds but was limited to males. Male sparrows were caught at the field site in Orleans County, Vermont, and were bled, injected, and bled again as described above, except that separate sets of birds received the GnRH and saline treatments. All birds included in this experiment were caught between May 10 and May 27, 2005, before the first egg of the season. That early in the breeding season, most of the birds are relatively synchronized in their nesting attempts, and variation in baseline T levels between males should be reduced.

Statistical Analyses. Values of plasma T in all experiments (except the field GnRH trial) were not normally distributed (positively skewed) and were log transformed ($\log(T + 1)$ or $\ln T$) to correct for this. During the course of the field study, 53 males (33 WS, 20 TS) and 37 females (13 WS, 24 TS) were sampled. Some individuals were sampled more than once over the course of the field study, but no individual was bled more than once in any 7-d period, to minimize the physiological

effects of sampling. A total of 94 samples were collected from males, with a mean of 1.8 samples per individual (range 1–7). Forty-three female samples were collected, with a mean of 1.4 samples per female (range 1–3). Field samples obtained from males and females were analyzed separately. T levels were compared between morphs with mixed-effects models constructed in SPSS 15.0 (SPSS 2007) or SAS (proc MIXED, SAS 9.1.2; SAS Institute 1994). Models were constructed with a backward-elimination strategy, and individual was entered as a random effect.

Our analysis also considered the effects of phenology. Date was converted to "corrected day," the Julian date on which the sample was taken corrected for the date that the first egg of that year was found in the study site. Using the corrected-day value allowed us to standardize our date variable across the three years of the study. This is advantageous because in 2004, breeding started approximately 9 d earlier than in 2003 or 2005.

A second analysis on the subset of birds whose nests we had monitored allowed us to examine the effect of nesting-cycle stage on differences in T between morphs. Because of sample size limitations, we grouped birds into two stages: "defense" and "parental." Females sampled during territory establishment, nest building, or lay (when females are fertile and soliciting copulations) were classified as "defense," whereas females captured during incubation, nestling feeding, and fledgling feeding were considered "parental." The definition of the defense and parental stages differed slightly in males because males of this species do not incubate or feed incubating females (Falls and Kopachena 1994) and therefore are not engaged in parental behavior during incubation. Males were classified in the "defense" stage when sampled during territory establishment, nest building, lay, or incubation and were classified as "parental" only when they were feeding nestlings or fledglings. Males ($n = 46$ individuals, 74 samples) and females ($n = 31$ individuals, 38 samples) were analyzed separately. T levels were analyzed by means of a mixed-effects model with morph and stage as fixed factors and individual as a random effect.

Results of the laboratory GnRH challenge experiments were analyzed with repeated-measures ANOVAs in the statistical software package JMP 5.0.1. Data from the GnRH challenge performed in the field were analyzed with a two-tailed t-test (GraphPad Prism 4.00).

Enzyme Immunoassay. White-throated sparrow plasma T levels were measured with an enzyme immunoassay kit from Assay Designs (catalog no. 900-065). These kits use raw plasma, which is added directly to the well. Steroid-binding globulins, which may interfere with assay reactions, can be degraded by adding a steroid displacement buffer (SDB). Because these kits are designed to be used with a variety of biological fluids, plasma dilution and concentration of SDB must be optimized. Optimization for T was performed in a manner similar to the optimization for corticosterone, as detailed in Wada et al. (2007). For optimization, a sample of pooled white-throated sparrow plasma was stripped of endogenous T by incubating plasma with a charcoal solution (1% Norit A charcoal and 0.1% dex-

tran in assay buffer). This stripped plasma was then spiked with a known concentration of T (500 pg/mL). Spiked stripped plasma was assayed at four dilutions (1 : 5, 1 : 10, 1 : 20, and 1 : 30, diluted with assay buffer) with three concentrations of SDB (0%, 1%, and 2%) and compared to a standard curve on the same plate. Hence, each sample should read at 500 pg/mL T unless there is interference from the plasma. For T, a plasma dilution of 1 : 20 with no SDB added removed the interference of plasma compounds in the assay.

Individual plasma samples were thawed, picofuged, vortexed, and diluted with assay buffer to a 1 : 20 concentration. Samples were aliquotted into separate wells in triplicate. The six-point standard curve (2,000–8.2 pg/mL) and a separate external standard were also run in triplicate on each plate. Enzyme-labeled T and antibody were added, and the plate was incubated at 26°C on a shaker for 2 h. Wells were then emptied and rinsed with wash buffer, and enzyme substrate was added. The plate was incubated for 1 h, again at 26°C, but without shaking. Stop solution was added after this final incubation, and the plate was immediately read with a Multiskan Ascent microplate reader at 405 nm, corrected at 595 nm. The lower limit of detectability for these assays was 1.6 pg per well, and all non-detectable samples were assigned this value. Samples were distributed randomly in regard to morph and sex, but not year, across 18 plates (nine plates of field samples and nine plates of captive samples). External standards were used to calculate interplate variability (11.6%), and intra-assay coefficients of variation were calculated from sample replicates (9.4%).

Results

Testosterone

Time of Year/Season. The linear mixed-effects model revealed a significant effect of the fixed factors morph ($P = 0.02$) and date ($P = 0.003$; Table 1). WS males had higher T than TS males, and levels declined over the breeding season in both morphs.

There was no significant difference in T between WS and TS females sampled in 2003 and 2004. The linear mixed-effect model (Table 1) revealed no significant effect of the fixed factor

Table 1: Testosterone in relation to morph and corrected day

Fixed Effect	Estimate	Standard Error	F	df	P
Males:					
Intercept	.874	.056	…	…	…
Morph	−.202	5.705	5.88	40	.019
Corrected day	−.013	9.065	9.46	40	.003
Females:					
Intercept	.3105	.06048	…	…	…
Morph	.05109	.06408	.64	11	.44
Corrected day	−.00243	.001191	4.15	11	.066

morph ($P = 0.44$). T declined with day in both morphs; however, this trend was not significant ($P = 0.07$).

Reproductive Substage. WS males had higher T levels than TS males in both stages ($P = 0.023$; Table 2; Fig. 1). T tended to be higher in both morphs during the defense stage, although this effect was not significant ($P = 0.12$; Table 2) There was no effect of morph in females (Table 2; Fig. 2), although, as in males, T was significantly higher during the defense stage. Note, however, that small sample sizes make our power to detect differences between female morphs in the defense stage very low. The lack of difference between WS and TS females during this stage should be interpreted with caution. There was no interaction between morph and stage in either males or females.

  Playback. Social interactions may increase plasma T in some species (Wingfield et al. 1990; Wingfield and Hahn 1994). Thus, longer song playbacks (a simulated social interaction) may influence T level. However, in this study, the use of playback to capture birds did not increase T in male white-throated sparrows (length of playback defined as time from start of playback to capture in the net). A linear regression revealed a loose but significant negative relationship between length of playback and plasma T levels (adjusted $r^2 = 0.06$, $b = -0.13$, $P = 0.04$). The negative slope of the correlation suggests that birds with higher endogenous T may have responded to playback and been captured more quickly than those with lower T, that is, that T was influencing time to capture, not that length of playback was influencing T within the sampling time frame. Alternatively, Landys et al. (2007), who recently demonstrated a similar negative relationship between plasma T and the length of playback, suggest that the absence of a plasma T increase in response to social challenge may allow single-brooded birds to avoid the detrimental effects of T on essential parental care behaviors (see also Van Duyse et al. 2004). This is a less convincing argument in our case because white-throated sparrows in this population may raise two broods per season. Regardless of the cause, the negative relationship between plasma T and length of playback should not bias the results here because there was no difference in the length of playback needed to capture birds of the two morphs ($n = 51$, $F = 1.18$, $P = 0.3$).

GnRH Challenge

Lab. Both males ($n = 21$: 14 WS, 7 TS; Fig. 3) and females ($n = 10$: 5 WS, 5 TS; Fig. 4) showed elevated T in response to

Table 2: Testosterone in relation to morph and nesting stage

Fixed Effect	Numerator df	Denominator df	F	P
Males:				
Morph	1	37	5.622	.023
Stage	1	68	2.495	.119
Females:				
Morph	1	27	.44	.51
Stage	1	1	5.25	.01

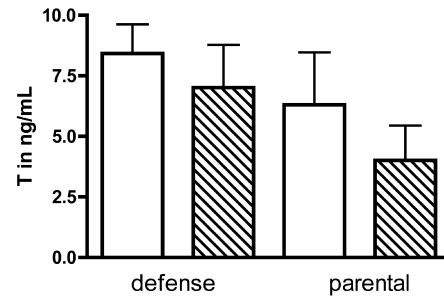### Male testosterone in relation to stage of nesting cycle

Figure 1. Plasma testosterone (T) in males during defense and parental stages of nesting cycle. Hatched bars represent mean T in tan-striped (TS) males; white bars represent mean T in white-striped (WS) males. Error bars represent SEM. The effect of morph, but not stage, was significant, according to the model. WS males had significantly higher T than TS males in both the defense and parental stages. T tended to be higher during the defense stage in both morphs. Sample sizes: WS defense = 30, TS defense = 26, WS parental = 8, TS parental = 9.

GnRH injection, compared to those injected with saline ($P < 0.001$ and $P = 0.003$, respectively). However, there was no difference between morphs in either sex (males: $P = 0.99$; females: $P = 0.48$).

  Field. We tested response to GnRH in free-living, breeding male white-throated sparrows ($n = 13$: 7 WS, 6 TS) to ensure that the lack of morph difference observed in the lab was not due to a lack of environmental cues. As in the lab, there was no difference between morphs in response to GnRH injection (Fig. 5; $t = 0.88$, df = 11, $P = 0.4$).

Discussion

Testosterone

This study demonstrates a significant difference in plasma T levels between WS and TS males during the breeding season. These differences in plasma T are consistent with morph-specific differences in aggression, suggesting that T may relate to this difference in male behavior. We have also demonstrated that WS males have higher T during both the defense and parental stages of the nesting cycle. Given the negative effects of T on parental behavior in other species (Schoech et al. 1998; Van Roo 2004; Schwagmeyer et al. 2005), it is possible that the higher T detected in WS males may relate to their reduced parental care.

  It must be emphasized that the magnitude of the difference in plasma T between male morphs is small. In all analyses, models indicated that the difference between morphs was between 0.5 and 0.6 ng/mL. The biological relevance of such a small amount of T is unclear. Individual variation in T levels, and thus variation within morphs, was high and certainly decreased the magnitude of the difference we detected. This vari-

Female testosterone in relation to stage of nesting cycle
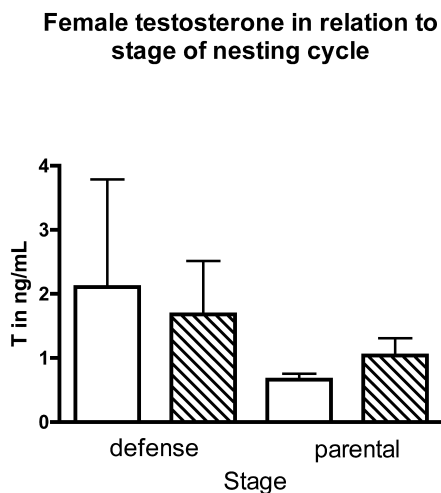

Figure 2. Plasma testosterone (T) in females during defense and parental stages of nesting cycle. Hatched bars represent mean T in tan-striped TS females; white bars represent mean T in white-striped WS females. Error bars represent SEM. The effect of stage was statistically significant, according to the model, but that of morph was not. T was significantly higher during the defense stage in both morphs; however, there was no significant difference between morphs. Sample sizes: WS defense = 3, TS defense = 5, WS parental = 12, TS parental = 21.

ability is to be expected because an individual's "testosterone phenotype" results from an interaction between its genotype and the environment. Factors in both the developmental and immediate social environment may modify the testosterone phenotype (Wingfield 1985). These factors are not expected to vary systematically with morph type and therefore will add noise to any morph-specific pattern in T. It should be noted that Formica et al. (2004) found that WS males settled in areas with a higher density of territories and therefore had more neighbors than did TS males. More neighbors could lead to more frequent territorial interactions. However, we did not observe differences in territory density between WS and TS males in our study site.

Despite potential individual differences in developmental history and social environment, we still detected a consistent difference between male morphs. In a similar study, Spinney et al. (2006) also found similar (small) differences in T between free-living male morphs captured in May (breeding stage unknown). Taken together with these, our results suggest that differential regulation of T may play a part in mediating differences in behavior between male morphs. However, other systems are almost certainly involved. For example, Maney et al. (2005) recently demonstrated that the WS morph has more vasotocin innervation in brain areas associated with agonistic behavior. Vasotocin is a neuropeptide hormone that has been associated with aggressive and courtship behavior in some species (Maney et al. 1997; Goodson et al. 2004). Gonadal steroids, including T, also modulate activity of the vasotocin neurons themselves and have been shown to have organizational as well

as activational effects on vasotocin immunoreactivity in the brain (Panzica et al. 2001).

Contrary to our prediction, we found no difference in T between WS and TS females, although our power to detect differences in T levels between female morphs during the defense stage was low and such a difference should not be ruled out. At this point, we have no evidence suggesting that circulating T concentrations mediate the difference in aggression between female morphs. Mechanisms of female aggression are poorly understood, and many studies have found no relationship between T and aggression in females (Elekonich and Wingfield 2000; Goymann and Wingfield 2004). However, in the dunnock (*Prunella modularis*), females involved in repeated aggressive interactions in competition for mates exhibited higher T levels than less aggressive females (Langmore et al. 2002). Similarly, female buff-breasted wrens (*Thryothorus leucotis*) exhibit elevation in T levels after simulated territorial intrusions during the prebreeding (defense) stage (Gill et al. 2007). It is possible that differences in aggression between WS and TS females may result from differences in other hormones, such as estrogen or progesterone, as suggested by work in reptiles and mammals (Kapusta 1998 [*Clethrionomys glareolus*]; Woodley and Moore 1999; Woodley et al. 2000 [*Sceloporus jarrovi*]). Or aggression may even be influenced by the ratio between two hormones such as T and progesterone, as, for example, in the mouse *Peromyscus californicus* (Davis and Marler 2003).

GnRH Challenge

Male morphs differ in plasma T levels, but what physiological difference between the morphs leads to this difference in T? The GnRH challenge can help localize the mechanism that leads to differences in T secretion. In this experiment, the pituitary was stimulated with a standard dose of exogenous GnRH, thus activating the pituitary-gonadal axis to secrete T. A difference
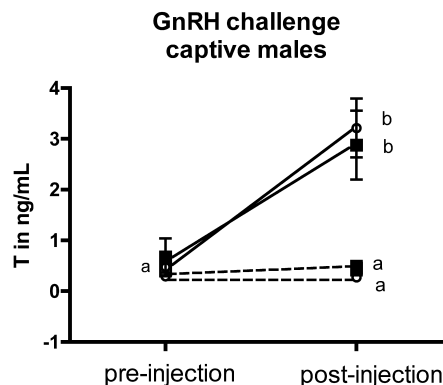
GnRH challenge captive males

Figure 3. Response of captive white-striped (*squares*; n = 14) and tan-striped (*circles*; n = 7) males to gonadotropin-releasing hormone (GnRH; *solid lines*) or saline (*dashed lines*) injections. Data are plotted as mean ± SEM. GnRH significantly increased T levels but did so equally in both morphs.
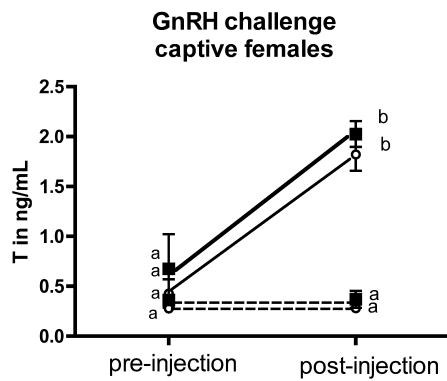
GnRH challenge captive females

Figure 4. Response of captive white-striped (*squares*; $n = 5$) and tan-striped (*circles*; $n = 5$) females to gonadotropin-releasing hormone (GnRH; *solid lines*) or saline (*dashed lines*) injections. Data are plotted as mean ± SEM. GnRH significantly increased T levels but did so equally in both morphs.

in T secretion between individuals indicates that their pituitaries or gonads differ in ability to respond to GnRH.

We found no difference between morphs in response to GnRH injection. The lack of difference in this study suggests that the morph difference in plasma T stems from a difference in T regulation at the hypothalamus or in higher brain regions rather than from a difference in the pituitary or gonad. Our findings are also consistent with those of Spinney et al. (2006), who found no differences between morphs in luteinizing hormone levels after GnRH injection. In contrast, their study did report a greater elevation of T in response to GnRH in WS males. There are two methodological differences between our study and the Spinney et al. (2006) study that may have led to the discrepancies between our results. First, Spinney et al. used a jugular injection of GnRH rather than an intramuscular injection. It is possible that injection method may have affected response to GnRH, but there is no a priori reason to expect such an effect. Second and more important, the birds used in the Spinney et al. study were captives housed communally outdoors, whereas we used both captives (housed indoors and individually) and free-living birds. However, both our study and Spinney et al.'s found comparable levels of variation in response to GnRH treatment (based on comparisons of standard errors), and the possible effects due to differences in housing (captive vs. free living, individually caged vs. communal) remain open to speculation. From the results of both studies, it is not entirely clear at which level in the hypothalamic-pituitary-gonadal axis differences in T are generated. Potential hypothalamic mechanisms include morph-specific differences in the number of T receptors involved in negative feedback or the number of GnRH-secreting neurons. Indeed, a recent study by Lake et al. (2008) found differences between WS and TS females in the number and size of GnRH-immunoreactive neurons in the hypothalamus.

Conclusion

This study demonstrates a difference in plasma T that correlates with the observed differences in aggression between male white-throated sparrow morphs. Furthermore, we found that this difference persists during both the defense and parental phases of the nesting cycle, suggesting that T may also be involved in differences in parental care. Our results suggest that the mechanism responsible for this difference in plasma T may lie at or above the hypothalamus. It remains to be seen what mechanisms, hormonal or otherwise, underlie differences in aggression between female morphs. Our findings constitute an essential first step in the investigation of the endocrine mechanisms responsible for generating a behavioral polymorphism in the white-throated sparrow. This species is an interesting case study in and of itself, but it is our hope that it will also provide a useful model system in which to compare and contrast distinct behavioral phenotypes within populations.
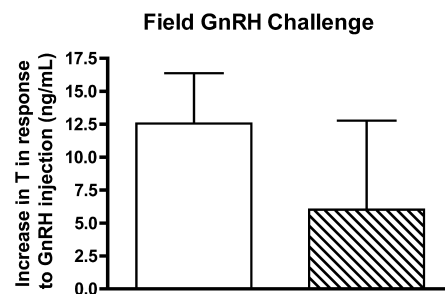
Field GnRH Challenge

Figure 5. Increase in plasma testosterone (T; plasma T after injection minus plasma T before injection) in free-living males treated with exogenous gonadotropin-releasing hormone (GnRH). Bars indicate mean + SEM. There was no significant difference between morphs (*white bar*: white-striped males, $n = 7$; *hatched bar*: tan-striped males, $n = 6$). Power analysis indicates that a sample size of 92 individuals per morph would be needed to detect a difference in response between morphs with $\beta = .10$.

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