

New insights into the hormonal and behavioural correlates of polymorphism in white-throated sparrows, *Zonotrichia albicollis*



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The white-throated sparrow is a promising model for behavioural neuroendocrinology and genetics because behaviour and endocrine function may be linked to a chromosomal rearrangement that determines plumage colour. The notion that the two colour morphs, tan-striped (TS) and white-striped (WS), differ predictably in aggression and parenting has been widely accepted, despite conflicting evidence. It is also hypothesized that morph-typic behaviour is hormone-mediated, yet no field study has measured sex steroids and behaviour in the same birds. Here, we re-evaluate the TS and WS phenotypes, describe the conditions under which they differ and investigate relationships between sex steroids and behaviour. We report that (1) during territorial intrusions, WS males were more aggressive than TS birds, but this difference was restricted to singing; WS males sang more than TS males but showed identical levels of physical aggression. WS females sang more than TS females and were also more physically aggressive. (2) TS males provisioned young more frequently than did WS males, but only during first broods. The parental strategy of WS males was flexible, and during replacement broods, WS and TS males provisioned at equal rates. (3) Consistent with previous studies, we detected no morph difference in female provisioning. (4) Plasma testosterone and dihydrotestosterone were higher in WS males than in TS males during periods of peak territorial defence and during first broods; within breeding stages, male androgen levels were positively correlated with singing and negatively correlated with provisioning. Plasma oestradiol levels were higher in WS females than in TS females and higher during peak territorial defence; oestradiol levels tended to be positively correlated with singing. Overall, our results refine the TS and WS phenotypes, show that behavioural differences between them are restricted to periods with relatively high mating opportunity, and demonstrate an association between sex steroids and morph-typic behaviour. These results will inform future studies of this promising model.

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Polymorphic species in which discrete genetic variants show alternative behavioural phenotypes can provide valuable insights into the proximate and ultimate causes of behaviour. The white-throated sparrow is such a species, and it is rising as a promising model for studying the neuroendocrine and genetic bases of social and reproductive behaviours. Its unique natural history first became apparent when Lowther (1961, 1962) described plumage polymorphism, disassortative mating and behavioural polymorphism in this species. He reported that two colour morphs, tan-striped (TS) and white-striped (WS), occur in both sexes, that birds paired with the opposite morph and that WS birds were more territorial than TS birds. Thorneycroft (1966, 1975) later revealed that plumage polymorphism is genetically based and discrete; colour morph

segregates with a rearrangement of the second chromosome and is independent of age, sex and season. All WS birds have at least one copy of the rearranged chromosome (ZAL2^m), whereas TS birds are homozygous for the wild-type arrangement (ZAL2).

Behavioural studies over the next two decades demonstrated that TS and WS birds often differ in territorial aggression, parenting behaviour and mating strategy (Collins & Houtman, 1999; Formica & Tuttle, 2009; Knapton & Falls, 1983; Kopachena & Falls, 1993a, 1993b; Tuttle, 1993, 2003), which led to a consensus in the current literature that morphs show alternative reproductive strategies (Formica, Gonsler, Ramsay, & Tuttle, 2004; Formica & Tuttle, 2009; Maney, 2008; Tuttle, 2003). Multiple authors have since described a number of endocrine differences between the morphs (Horton & Holberton, 2009, 2010; Lake, Lange, O'Brien, Sanford, & Maney, 2008; Maney, 2008; Spinney, Bentley, & Hau, 2006; Swett & Breuner, 2008, 2009), suggesting that morph-typic strategies may be hormone-mediated. Most recently, genetic studies have revealed

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that the ZAL2^m rearrangement captures a suite of neuroendocrine genes (Thomas et al., 2008). The apparent linkage between the ZAL2^m rearrangement, endocrine activity and function, and reproductive strategy provides an exciting premise for targeted investigations of the neuroendocrine and genetic bases of behaviour (Horton et al., 2013; Horton et al., 2014; Maney, 2008; Maney, Erwin, & Goode, 2005).

A more thorough understanding of natural variation in white-throated sparrow behaviour and endocrinology is essential to the design and interpretation of future studies and key to capitalizing on the full potential of this unique natural model. Although numerous authors have examined the behaviour, physiology and ecology of this species (reviewed in Falls & Kopachena, 2010), relatively few have explicitly studied morph-dependent trait variation. Plumage morph is certainly discrete and linked to the ZAL2^m rearrangement (Thornycroft, 1966, 1975), but the evidence that behaviour is equally polymorphic and correlated with hormonal differences is less concrete. Indeed, other researchers have called for more investigations of individual variation in hormone–behaviour relationships (Ball & Balthazart, 2008; Kempenaers, Peters, & Foerster, 2008; Rosvall et al., 2012; Williams, 2008, 2012). In this study, we re-evaluated morph-dependent variation in territorial aggression, parental care and sex steroid levels in a free-living population. Our aims were to better define the natural behavioural phenotypes of TS and WS birds with respect to their alternative reproductive strategies and to examine more closely the endocrine correlates of morph-typic behaviour.

Reproductive Behaviour

Territorial aggression

Because aggressive interactions in songbirds typically occur in the context of territorial defence, aggression is often quantified by simulating a territorial intrusion with a model and song playback (Horton, Hauber, & Maney, 2012; Wingfield & Hahn, 1994). Multiple intrusion experiments on white-throated sparrows have demonstrated that resident WS birds of both sexes sing more in response to intrusions than do their TS counterparts (Kopachena & Falls, 1993a; Jones, 1987; Lowther, 1962). A more recent study, however, did not replicate this finding (Collins & Houtman, 1999), and studies of nonvocal aggression have not always demonstrated morph differences (Kopachena & Falls, 1993a; Lowther, 1962). Simulating the most threatening conditions possible is necessary to properly define aggressive phenotypes and predict their fitness consequences (Gross, 1996); however, no published study of this species has examined morph-dependent aggression during the peak time of aggression using the strongest combination of stimuli. Here, we challenged resident birds with both live decoys and song playback at a time when territories were unstable and females were receptive; under these conditions, territorial defence is maximal (Falls & Kopachena, 2010).

Parental care

The best-studied aspect of parental care in white-throated sparrows is nestling provisioning behaviour. Two classic studies demonstrated that although the morphs do not differ in the number, size or types of items fed to nestlings, TS birds of both sexes provision at higher rates than do their WS counterparts (Knapton & Falls, 1983; Kopachena & Falls, 1993b). Little attention has been paid, however, to the fact that three other studies failed to detect a morph difference in provisioning rate in one or both sexes (Horton & Holberton, 2010; Kopachena & Falls, 1993c; Whillans & Falls, 1990). Thus, understanding polymorphic parental behaviour in this species may require more thoughtful consideration of the conditions under which it occurs.

One of the most important factors to consider is timing, in other words the stage of the breeding season in which a test is conducted. In songbirds, the degree of parental investment can increase as the breeding season progresses (Biermann & Robertson, 1981; Robinson, Siefferman, & Risch, 2010). In white-throated sparrows, territorial boundaries become more stable and extrapair mating opportunities decline (Falls & Kopachena, 2010). Because investment in territorial aggression and mate-seeking behaviours depend on morph (Formica & Tuttle, 2009; Tuttle, 1993, 2003), TS and WS birds may differ in the way they modify parental effort as the breeding season progresses. In this study, we compared the overall levels of parental effort shown by each morph and sex, tested whether parental birds altered their provisioning rates as the season progressed, and determined the extent to which these changes depended on morph.

Endocrine Correlates of Polymorphism

In birds, territorial and parental behaviours are tightly linked to endocrine state. Many authors have therefore hypothesized that morph differences in behaviour may be mediated by the endocrine system (reviewed by Maney, 2008; also see Horton & Holberton, 2009, 2010; Swett & Breuner, 2009). Morph differences in behaviour are evident during spring and summer, when gonads are recrudescing (reviewed by Maney, 2008), but not during autumn or winter, when gonads are regressed (Piper & Wiley, 1989, 1990). That morph-typic behaviour is seasonal suggests an important role for sex steroids. Two studies of free-living birds revealed morph differences in male plasma testosterone (T) levels during the breeding season; in both cases, WS males had higher T levels than TS males (Spinney et al., 2006; Swett & Breuner, 2009). Although intriguing, these studies provide little evidence that behaviour is related to T levels in this species because behaviour was not measured in either. Morph differences in T levels may not be ubiquitous (see DeVoogd, Houtman, & Falls, 1995), and despite the apparent consistency of morph differences in female territorial aggression, female T levels have not been shown to vary according to morph (Spinney et al., 2006; Swett & Breuner, 2009). It thus remains to be shown whether behavioural polymorphism in this species is associated with plasma levels of sex hormones.

In this study, we measured plasma levels of T, dihydrotestosterone (DHT) and oestradiol (E2) during multiple stages of the breeding season in free-living white-throated sparrows. Plasma steroid levels may depend not only on morph, but also on breeding stage (Horton & Holberton, 2010; Swett & Breuner, 2009), and little attention has been paid to how these factors interact (DeVoogd et al., 1995; Spinney et al., 2006; but see Horton & Holberton, 2010; Swett & Breuner, 2009). Thus, we examined whether hormone levels differed between the morphs within and across breeding stages in a manner consistent with morph differences in behaviour. Individual variation in hormone levels is underutilized in field endocrine studies, even though it may predict variation in the behaviours that these hormones are expected to regulate (Kempenaers et al., 2008; Rosvall et al., 2012; Williams, 2008). Therefore, for a subset of birds, we collected behavioural data and hormone samples concurrently to test whether sex steroid levels were correlated with territorial and/or parental behaviours.

METHODS

Study Population

We studied white-throated sparrows during the 2010 and 2011 breeding seasons in the Hemlock Stream Forest in Bradley, Maine, U.S.A. (45°5'34.2"N, 68°41'23.2"W; elevation: 30 m). Our study

population consisted of nearly equal numbers of the two most common pair types: WS male \times TS female and TS male \times WS female. One same-morph pair (a WS \times WS) was observed in this population, but it was not included in this study. Territories held by the two pair types were interspersed evenly throughout the site. We conducted the study from early May to late July during the prelaying, laying, incubation and nestling stages. We quantified territorial aggression during the early breeding stages and quantified parental care during the nestling stage. Territorial aggression was studied at the southern end of the site, whereas parental care was studied at the northern end. With few exceptions, observations of aggression and parenting behaviour were conducted on different breeding pairs.

Ethical Note

All research was carried out with the approval of the Emory University Institutional Animal Care and Use Committee (protocol number 048-2010) and with the appropriate state (Maine Inland Fisheries and Wildlife, permit numbers 2010-295 and 2011-295) and federal (U.S. Geological Survey, USGS permit number 23369; U.S. Fish and Wildlife Service permit number MB009702) permits for banding and scientific collection. Moreover, all procedures, including banding, blood sampling and behavioural studies, adhered to the Guidelines to the Use of Wild Birds in Research (Gaunt & Oring, 1999). To our knowledge, none of the procedures described below led to the abandonment of territories or nests. As described below, some birds were collected and tissues were harvested for use in another study; euthanasia was performed quickly (<1 min) by isoflurane overdose. Tissues were harvested in the field, and birds were not held in captivity before collection. Permission to conduct this research on the Hemlock Stream Forest was granted by the Forest Society of Maine.

Territorial Aggression

We studied territorial aggression during 12–31 May in both years. This sampling period followed pair formation and territory establishment, but generally preceded incubation in the population. During this phase of the breeding cycle, a large proportion of females in the population are presumed to be fertile (Birkhead & Møller, 1992), and both mating effort and territorial defence escalate to peak levels (Falls & Kopachena, 2010; Tuttle, 2003). Between 3 and 11 days before the behavioural tests began, we captured birds by luring them into mist nets with conspecific song playback. Nets were watched at all times to record exact capture times. We recorded the duration between the start of playback and capture (or playback time) for each bird; most birds were captured rapidly (mean \pm SE = 4.5 ± 0.4 min; range 0.25–20 min). After capture, a blood sample (150 μ l) was collected in a heparinized capillary tube following puncture of the brachial vein with a 26-gauge needle. All blood samples were collected within 8.5 min of capture (mean \pm SE = 3.3 ± 0.1 min) to minimize the effects of capture stress on sex steroid levels (Deviche et al., 2010). Blood-sampling times did not differ between morphs for males ($t_{1,73} = 0.96$, $P = 0.34$) or females ($t_{1,42} = 0.48$, $P = 0.64$). Blood samples were kept on ice until centrifugation, usually within 6 h, after which plasma was removed and kept frozen until sex steroid assays (described below). After blood sampling, we tagged each bird with an aluminium USGS identification band and a unique combination of coloured leg bands for identification during behavioural tests. For males, we calculated the volume of the cloacal protuberance using the formula $V = \pi a^2 b$; where a is half the width of the cloacal protuberance at its widest point and b is the height of the cloacal protuberance from its base (Mulder & Cockburn, 1993). For females, we scored brood patch

development. All birds for the aggression study were banded during the prelaying stage, confirmed in some cases by the lack of brood patch development in the female, which does not begin until nest building (Falls & Kopachena, 2010), and confirmed in other cases by observing nest-building behaviour by the female.

Behavioural assays

We measured territorial aggression by performing simulated territorial intrusions. To perform the test, we placed a live, caged male decoy centrally in a known territory and broadcasted conspecific song (playback) from an LG[®] portable stereo speaker placed next to the cage and controlled remotely with an Apple iPod Nano[®]. In this species, a decoy accompanied by playback elicits a stronger territorial response than does a decoy or playback alone (Jones, 1987). Each of 20 unique decoys (10 WS, 10 TS) was presented to no more than two pairs within a pair type. Twelve song exemplars were downloaded from the Borror Laboratory of Bioacoustics and edited according to Maney et al. (2009) to equalize volume and eliminate background noise. These exemplars varied according to recording locale, pattern of ascending versus descending syllables, and pitch. Each year, decoys were randomly assigned a song exemplar; songs were used for only one decoy each year. Playback during simulated territorial intrusions consisted of the one song repeated every 15 s.

Decoy morph can influence territorial responses in this species (Kopachena & Falls, 1993a; Horton et al., 2012; Jones, 1987). Thus, we performed two simulated territorial intrusions on each territorial pair, presenting a TS male decoy for one simulated territorial intrusion and a WS male decoy for the other such that the order of presentation was counterbalanced. The two simulated territorial intrusions for each pair were conducted on consecutive days at the same time of day (0600–1130 hours Eastern Daylight Time, EDT), but we balanced the time of day across pairs so that both pair types received earlier and later simulated territorial intrusions. We did not conduct simulated territorial intrusions on immediate neighbours on the same day. Each simulated territorial intrusion was monitored by two observers positioned 30 m apart and on opposite sides of the decoy.

Once playback commenced, we recorded the latency to approach, defined as the time from playback start until the resident arrived to investigate the decoy. After the resident male arrived, we conducted the simulated territorial intrusion for an additional 10 min. In addition to approach latency, we recorded behaviours indicative of territorial aggression in this species (Collins & Houtman, 1999; Jones, 1987; Kopachena & Falls, 1993a). These included four physical behaviours (number of flights directly over the decoy, time spent within 5 m and within 2 m of the decoy and the distance from the decoy at closest approach) and seven types of vocalizations (chip (pink) calls, chip-up calls, trills, soft songs, partial songs and full songs; for descriptions of each type of vocalization, see Falls & Kopachena, 2010). Both male and female white-throated sparrows show all of these behaviours in response to simulated territorial intrusion (Falls & Kopachena, 2010; Horton et al., 2012; Jones, 1987; Kopachena & Falls, 1993a), so we scored all of the behaviours for both members of each pair. Females may also perform a copulation solicitation display (Falls & Kopachena, 2010), but only one female in this study solicited a male decoy. Thus, female responses in this study, like those of males, were largely aggressive in nature.

We collected a large subset of the birds to harvest their tissues for another study. Each bird in this subset was captured on the day following its last simulated territorial intrusion and blood was collected as described above. During tissue harvest, we again measured male cloacal protuberance volume, and we calculated the volume of each testis using the formula $V = 4/3\pi a^2 b$; where a is

half the testis' width at the widest point and b is half its length along the long axis (Spinney et al., 2006; Wingfield et al., 2003). The breeding stage of each bird was reassessed at the time of collection; for example, the prelaying stage was confirmed by the lack of a brood patch in the female, and the laying stage was confirmed by the presence of eggs in the female's oviduct or shell gland.

Statistics

The context of behavioural responses to simulated territorial intrusion differed between males and females because all intruders were male; thus, we analysed the data from males and females separately. For every individual, we used the data from the two consecutive simulated territorial intrusions to calculate the mean number of times that each behaviour was performed. These means were then square-root transformed to yield normal residuals. One of our primary aims was to better define the aggressive phenotypes of the two morphs, and to do this we needed to consider aggressive behaviours individually and not as part of a combined aggression score. Thus, we used Student's t tests to look for morph differences in each of the behaviours separately, and we did not correct for familywise error rates. P values depicted in Tables 1 and 2 should be viewed accordingly.

Parental Behaviour

We conducted the parental behaviour study each year during June and July. Nests were found either by flushing incubating or brooding females, or by observing nesting behaviour. Once located, nests were monitored every other day until hatching. We captured parental birds during the incubation stage or within 2 days of their eggs hatching to band them for identification during behavioural data collection (see below). We captured parental birds in mist nets either by luring them with conspecific song playback or by passively capturing them in nets placed near the nest. Banding, measuring and blood sampling were conducted as described above. The identity of each parent was confirmed by observing its parental behaviour at the nest, either with binoculars or on video.

Behavioural data collection

We used video cameras (Sanyo® Xacti, HD, 30x optical zoom) to record parental behaviour at the nest (Horton & Holberton, 2009, 2010). When weather permitted, nests were filmed for 3 continuous hours between 0530 and 1130 hours EDT on 2 consecutive days when nestlings were 5 and 6 days old. The time of day at which nests were filmed did not differ between pair types ($t = 0.29$, $P = 0.77$). Cameras covered with camouflage cloth were concealed among standing vegetation 3–5 m from the nest. Behaviour and vocalizations monitored on video suggest that the vast majority of parents were either not disturbed by cameras or adjusted rapidly (<10 min) to them; behaviours were not scored during this brief adjustment period. If there were signs that the camera's presence disrupted parental behaviour (e.g. continued alarm calling or lack of feeding visits) for an extended period (i.e. >20 min), we excluded the entire video from analyses.

The morphs have been shown to differ in the rate at which they provision young, but not in the number or size of items that they deliver per trip (Knapton & Falls, 1983). During video transcription, therefore, we recorded the provisioning rate (number of feeding trips/h) by each parent. We also recorded brooding time (min/h) for females; according to our video data, males rarely brood in this population (also see Falls & Kopachena, 2010). Lastly, we recorded the rate of spontaneous singing (songs/h) by parental males. In most cases, we could clearly hear the territorial male's song on the audio; song data were excluded if songs by the focal male were indistinguishable from those of his neighbours, or if background

noise prevented accurate quantification of the focal male's song rate. Parental females rarely sing (B. Horton, personal observation) and their songs were not considered in this analysis.

On nestling day 7, we collected a large subset of the observed birds for another study. We captured them using the same techniques used to capture them for banding. We collected blood samples from the birds prior to sacrifice and measured cloacal protuberance and testis volume during tissue harvest as described above for the aggression study.

In white-throated sparrows, reneating is common because of high nest predation rates; first broods are highly synchronous, whereas replacement broods occur asynchronously (Falls & Kopachena, 2010). In this study, we observed parental behaviour during first broods and replacement broods (or reneats). Double brooding does occur in this species (Falls & Kopachena, 2010), but second broods were not included in this study. Only one brood was observed for each breeding pair. During both years of this study, young in first broods fledged during 12–25 June and young in replacement broods fledged after 4 July, and sporadically thereafter. We included nesting attempt in our analyses below to examine whether parental investment (and hormone levels) varied across broods.

Statistics

For every individual, we used behavioural data from days 5 and 6 of the nestling period to calculate a mean provisioning rate for each parent, mean spontaneous song rate for males and mean brooding rate for females; these means were then square-root transformed to yield normal residuals. We analysed male and female data separately because behaviour during the nestling stage is sex dependent (Falls & Kopachena, 2010), and because we were most interested in morph differences in behaviour within the sexes. To examine the overall effects of morph and nesting attempt on behaviour, we used mixed-model ANCOVAs with morph and nesting attempt (two levels) as fixed effects, nestling number as a covariate and year as a random effect. To examine whether a particular morph altered their parental behaviour across nesting attempts, we used t tests to compare behaviour between nesting attempts within each morph. To test whether morph differences in behaviour depended on when birds were sampled, we used t tests to compare behaviour between the morphs within each nesting attempt. To determine whether singing was correlated with provisioning effort in males, we used the mean for each behaviour to conduct a regression analysis.

Endocrine Correlates of Morph and Behaviour

Hormone assays

We measured sex steroid concentrations in samples collected prior to behavioural observations (i.e. during banding) and after behavioural studies (see above). Plasma T, DHT and E2 were assayed according to the procedures of Stevenson, Small, Ball, and Moore (2012). Briefly, plasma samples were fractionated by column chromatography to separate sex steroids and analysed by radioimmunoassay. We used the same antibody (T-3003, Wigen Laboratories, Succasunna, NJ, U.S.A.) to assay T and DHT after fractionation; E2 levels were assayed with a different antibody (7010-2650-serum, Biogenesis Ltd, Dorset, U.K.). Hormone concentrations were corrected for individual extraction efficiencies; average recoveries were 70% for T, 30% for DHT and 48% for E2. Samples were run in two assays, and samples from 2010 and 2011 were randomly allocated to each assay. Interassay variation was 10.2% for T, 9.1% for DHT and 5.5% for E2. Intra-assay variations were 13.4% and 14.2% for T, 9.4% and 9.1% for DHT, and 4.1% and 13.1% for E2. Lower detectable limits were 0.11 ng/ml for T, 0.30 ng/ml for

DHT and 0.11 ng/ml for E2. If T, DHT or E2 levels in a given sample were below the assay's limit of detection, the hormone concentration was entered as the lowest detectable limit. In males, 99% of samples had detectable T levels, 79% had detectable DHT levels and 47% had detectable E2 levels. In females, T levels were detectable in 82% of samples, whereas DHT and E2 levels were detectable in 32% and 41% of samples, respectively.

Statistics

To test whether sex steroid profiles across the breeding season were morph dependent, we compared hormone levels in birds across five breeding stages. All prelaying, laying and incubating birds were in their first nesting attempt of the season. The nestling stage was divided into two substages, first broods and replacement broods, to test for hormonal changes across nesting attempts (Logan & Wingfield, 1995). Note that female parental care begins with incubation, but males are not parental until the nestling stage (Falls & Kopachena, 2010). Only hormone data from individuals in known stages were used in these analyses. Sex steroid levels and their effects on behaviour are often sex dependent; thus, we analysed male and female hormone data separately.

To test for the main effects of morph and breeding stage on sex steroid levels, we analysed each steroid separately using mixed-model ANCOVAs. The models examined morph and stage as fixed main effects and the morph*stage interaction term as a fixed effect; each model included playback time as a covariate and year as a random effect. Male models also included individual ID as a nested random effect to control for repeat sampling (20% of male samples represent a resample during another stage); females were not resampled. Hormone data, which were non-normal, were transformed prior to ANCOVAs using the adjusted rank transformation (ART) method described by Leys and Schumann (2010). This nonparametric approach guards against non-normality and heteroscedasticity, and it is a more powerful test for main effects than traditional nonparametric analyses, and more appropriate than using ANOVA-based tests on non-normal data to test for interactions (Leys & Schumann, 2010; Mansouri, Paige, & Suries, 2004). To explicitly test the prediction that the ability to detect morph differences in steroid levels depends on when birds are sampled, we compared steroid levels (nontransformed data) between the morphs within each breeding stage using Wilcoxon two-sample tests with a χ^2 approximation.

To determine whether sex steroid levels were correlated with song and parental behaviour, we limited our analysis to the birds from which we took blood samples within 1 day after the behavioural tests (see above). We conducted regression analyses to test whether plasma levels of sex steroids were correlated with singing or nestling provisioning. To relate hormone levels and song, we used the mean number of songs (including all song types) over the two consecutive simulated territorial intrusions, or over days 5 and

6 of the nestling period for parental males. To relate hormone levels and parental behaviour, we used the mean provisioning rate during days 5 and 6 of the nestling period. These means were then regressed against hormone levels. Because hormones and behaviour in this species are dependent on morph and breeding stage (see Results), these effects alone can drive hormone–behaviour correlations. Thus, we used multiple regression analyses to parse out the relative influence of morph, stage and sex steroids (independent variables) on behaviour (dependent variable); we report partial regression coefficients and associated *P* values for each independent variable. Hormone data were square-root transformed for regression analyses, which yielded normal residuals.

We used data on cloacal protuberance and testis volume to re-evaluate prior, but inconsistent, reports of morph differences in both characteristics (DeVoogd et al., 1995; Maney, Lange, Raees, Reid, & Sanford, 2009; Spinney et al., 2006). We analysed cloacal protuberance and testis data using ANCOVAs with morph as a fixed effect, and Julian day and body mass as covariates.

RESULTS

Territorial Aggression

We conducted simulated territorial intrusions on 15 WS male \times TS female pairs and 15 TS male \times WS female pairs. Behavioural response data and comparisons across morph are summarized in Tables 1 and 2, and key results are graphed in Fig. 1. We found that the morphs differed in their aggressive responses to simulated territorial intrusions. In both sexes, WS birds responded with more full songs and a higher total song number (summed full, soft and partial songs) than did their TS counterparts (Table 1). WS and TS males did not differ in their physical responses, but WS females showed higher physical aggression than TS females. WS females displayed a shorter response latency, spent more time within 5 m of the decoy, and approached the decoy more closely than did TS females (Table 2, Fig. 1). Although TS females are not thought to sing or contribute substantially to territorial defence in this species, we observed three TS females singing during these simulated territorial intrusion experiments, and several TS females showed aggression towards intruders.

Parental Care

We analysed the parental behaviour of 9 WS male \times TS female pairs and 16 TS male \times WS female pairs. In males, nestling provisioning rate varied according to morph ($F_{1,25} = 7.3$, $P = 0.01$) and nest attempt ($F_{1,25} = 10.3$, $P < 0.01$); overall, TS males fed nestlings at higher rates than WS males, and males provisioned young in replacement broods at higher rates than those in first broods. The provisioning rates of WS males were considerably higher during

Table 1
Vocal responses of tan-striped (TS) and white-striped (WS) white-throated sparrows to simulated territorial intrusions

	Males				Females			
	TS (N=15)	WS (N=15)	Morph comparison		TS (N=15)	WS (N=15)	Morph comparison	
			<i>t</i>	<i>P</i>			<i>t</i>	<i>P</i>
Chips	7.0 \pm 1.9	8.7 \pm 2.3	−0.3	0.792	9.1 \pm 2.4	9.0 \pm 1.7	−0.4	0.678
Chip-ups	0.1 \pm 0.03	0.3 \pm 0.2	−1.0	0.329	0.2 \pm 0.1	0.5 \pm 0.3	−0.6	0.534
Trills	0.2 \pm 0.1	0.6 \pm 0.4	−0.8	0.424	2.9 \pm 1.0	5.2 \pm 1.0	−1.9	0.063
Full songs	3.6 \pm 1.0	16.0 \pm 3.2	−4.5	<0.001	0	2.9 \pm 1.6	−2.74	0.011
Soft songs	2.2 \pm 1.1	3.1 \pm 1.0	−0.9	0.360	0.6 \pm 0.5	1.0 \pm 0.4	−1.0	0.343
Partial songs	3.4 \pm 1.3	7.3 \pm 2.3	−1.9	0.067	0.9 \pm 0.5	2.5 \pm 1.3	−1.45	0.158
Total songs	9.2 \pm 2.4	27.3 \pm 4.0	−4.2	<0.001	1.5 \pm 0.9	6.4 \pm 2.3	−2.1	0.049

Means \pm SE are shown for males and females of each morph for each behaviour quantified, as well as *t* and *P* values for within-sex comparisons between the morphs. Significant *P* values are shown in bold.

Table 2

Physical responses of tan-striped (TS) and white-striped (WS) white-throated sparrows to simulated territorial intrusions

	Males				Females			
	TS (N=15)	WS (N=15)	Morph comparison		TS (N=15)	WS (N=15)	Morph comparison	
			<i>t</i>	<i>P</i>			<i>t</i>	<i>P</i>
Latency to respond (min)	1.1±0.3	1.1±0.2	−0.1	0.980	4.5±1.0	1.3±0.2	3.1	0.004
Time within 5 m (min)	7.0±0.8	8.0±0.7	−0.6	0.557	3.5±1.0	6.4±0.9	−2.3	0.032
Time within 2 m (min)	2.7±0.6	2.6±0.6	0.3	0.742	2.1±0.7	3.1±0.8	−1.12	0.273
Closest approach (m)	1.6±0.5	1.6±0.4	−0.2	0.881	9.2±2.2	2.3±0.7	2.79	0.009
Flights over decoy	7.5±2.0	7.2±1.5	−0.4	0.732	2.2±0.9	3.4±0.8	−1.44	0.162

Means ± SE are shown for males and females of each morph for each behaviour quantified, as well as *t* and *P* values for within-sex comparisons between the morphs. Significant *P* values are shown in bold.

replacement nests than during first nests ($t_{1,8} = 2.7$, $P = 0.03$); provisioning rates of TS males also tended to increase across nesting attempts, but not significantly so ($t_{1,15} = 1.9$, $P = 0.08$; Fig. 2a). Morph differences in male parental behaviour also depended on when the birds were sampled; the provisioning rates of TS and WS males were significantly different for first broods ($t_{1,12} = 2.3$, $P = 0.04$), but not for replacement broods ($t_{1,11} = 0.7$, $P = 0.49$; Fig. 2a). Spontaneous song rates in parental males also varied according to morph ($F_{1,19} = 14.6$, $P < 0.01$) and nesting attempt ($F_{1,19} = 4.6$, $P = 0.05$). Overall, WS males sang more often than TS males, but significantly so only during first broods (first brood: $t_{1,9} = 4.7$, $P < 0.01$; replacement brood: $t_{1,9} = 0.2$, $P = 0.86$; Fig. 2b). Song rates of WS males were higher during first broods than during replacement broods ($t_{1,6} = 3.1$, $P = 0.03$), whereas song rates of TS males were similar across broods ($t_{1,12} = 0.5$, $P = 0.65$). Indeed, male parental song rates and nestling provisioning rates were inversely correlated ($R^2 = 0.35$, $P < 0.01$; Fig. 2c).

In females, provisioning rate did not depend on morph ($F_{1,25} = 0.4$, $P = 0.56$), and provisioning effort did not vary according to nesting attempt ($F_{1,25} = 0.1$, $P = 0.99$; Fig. 2d). There was a morph difference, however, in brooding behaviour among females; overall, WS females brooded young at higher rates than did TS females ($F_{1,25} = 6.5$, $P = 0.02$; Fig. 2e). Females also reduced their brooding effort between first and replacement broods ($F_{1,25} = 23.3$, $P < 0.01$); this result may be explained by the warmer ambient temperatures that accompany replacement broods compared with first broods. WS females tended to brood their young more often than TS females during first broods ($t_{1,12} = 2.0$, $P = 0.08$) and during replacement broods ($t_{1,10} = 2.7$, $P = 0.03$; Fig. 2e).

Hormones

We collected a total of 121 plasma samples from males ($N = 77$) and females ($N = 44$) in known breeding stages over the course of

the study. Hormonal profiles and sample sizes by morph, sex and breeding stage are shown in Fig. 3.

Males

Morph differences in male plasma androgen levels depended on when birds were sampled (morph*stage effect: T: $P < 0.001$; DHT: $P = 0.004$; Table 3, Fig. 3a, b). Levels of both androgens were higher in WS males than in TS males during the prelaying (T: $\chi^2_{1,24} = 13.0$, $P < 0.01$; DHT: $\chi^2_{1,24} = 3.8$, $P = 0.05$) and incubation (T: $\chi^2_{1,13} = 4.3$, $P = 0.04$; DHT: $\chi^2_{1,13} = 3.3$, $P = 0.06$) stages, but not during laying (T: $\chi^2_{1,8} = 1.5$, $P = 0.22$; DHT: $\chi^2_{1,8} = 0.2$, $P = 0.62$). During the nestling stage, parental WS males had higher androgen levels than TS males during first broods (T: $\chi^2_{1,10} = 4.0$, $P = 0.04$; DHT: $\chi^2_{1,10} = 4.4$, $P = 0.04$) but not during replacement broods (T: $\chi^2_{1,7} = 0.4$, $P = 0.55$; DHT: $\chi^2_{1,7} = 0.1$, $P = 0.86$; Fig. 3a, b). Significant main effects of morph and stage (Table 3) also indicate that male androgen levels declined as breeding progressed and were higher in WS males than in TS males (Fig. 3a, b). Male E2 levels differed between the morphs ($P = 0.033$; Fig. 3c) and tended to decline as breeding progressed ($P = 0.068$; Table 3), but there was not a significant morph*stage interaction (Table 3). Nevertheless, the main effect of morph appeared to be driven by higher E2 levels in WS males than in TS males during the laying stage ($\chi^2_{1,8} = 4.8$, $P = 0.03$) and during first broods ($\chi^2_{1,10} = 5.2$, $P = 0.02$; Fig. 3c), as E2 levels were similar between the morphs during other stages (all $\chi^2 \leq 1.5$, all $P \geq 0.23$).

Females

Morph differences in female E2 levels depended on when birds were sampled (morph*stage effect: $P = 0.030$; Table 3, Fig. 3f). E2 levels were higher in WS females than in TS females during non-parental stages (prelaying: $\chi^2_{1,12} = 7.2$, $P = 0.007$; laying: $\chi^2_{1,6} = 3.9$, $P = 0.049$), but not during parental stages (all $\chi^2 < 3.9$, all $P > 0.05$). Female T levels varied according to morph ($P = 0.038$)

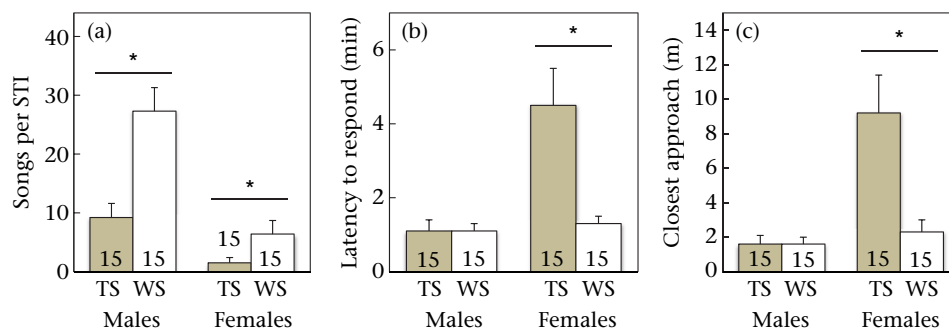


Figure 1. Aggressive behaviours by male and female white-throated sparrows in response to simulated territorial intrusions (STIs). (a) Song rate (a measure of vocal aggression). (b) response latency and (c) closest approach distance to the intruder (measures of physical aggression). TS: tan-striped morph; WS: white-striped morph. Values are means ± SE; numbers in bars denote sample sizes.

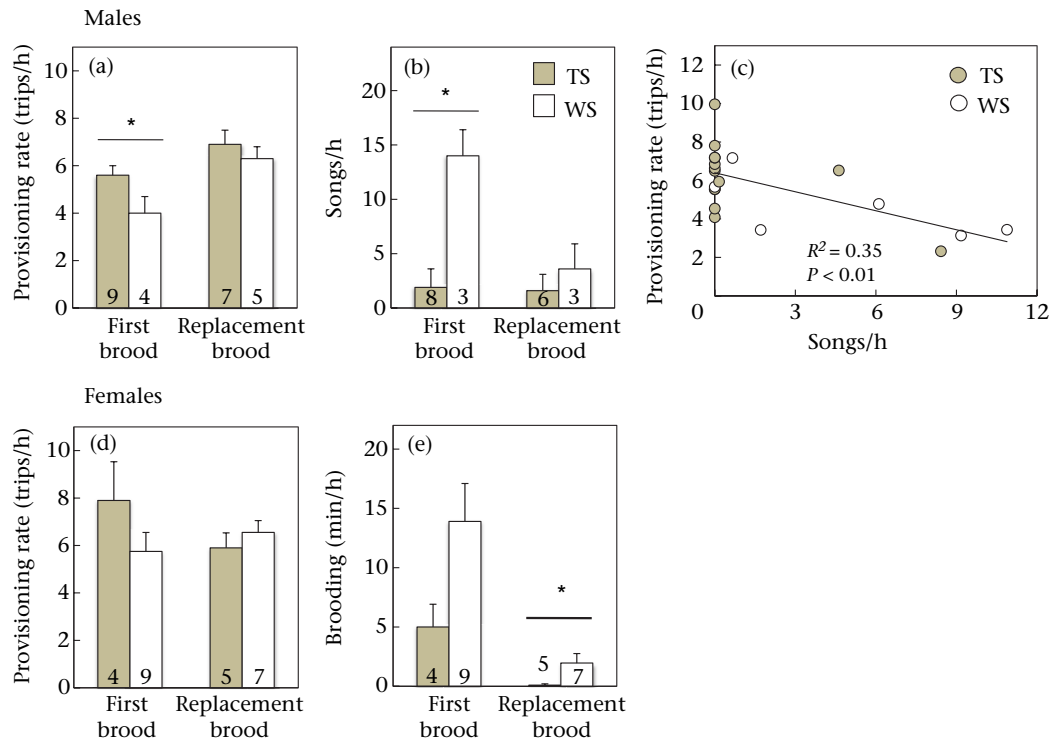


Figure 2. Behavioural polymorphism during the nestling stage in white-throated sparrows tending to first broods of the season or replacement broods. (a) Nestling provisioning rates, (b) spontaneous song rates and (c) the relationship between these two behaviours for parental males. (d) Nestling provisioning rates and (e) brooding time for parental females. TS: tan-striped morph; WS: white-striped morph. Values are means \pm SE; numbers in bars denote sample sizes.

but not breeding stage ($P = 0.251$; Table 3, Fig. 3d), but there was no interaction between these factors ($P = 0.513$; Table 3). Overall, T levels were higher in WS females than in TS females (Fig. 3d). Although T levels did appear to be higher in WS females than in TS

females during both nonparental stages and during first broods (Fig. 3d), we detected no significant morph differences in T within any stage (all $\chi^2 < 1.9$, all $P > 0.16$). It is possible that sample sizes for females, which were smaller than those for males, inhibited our

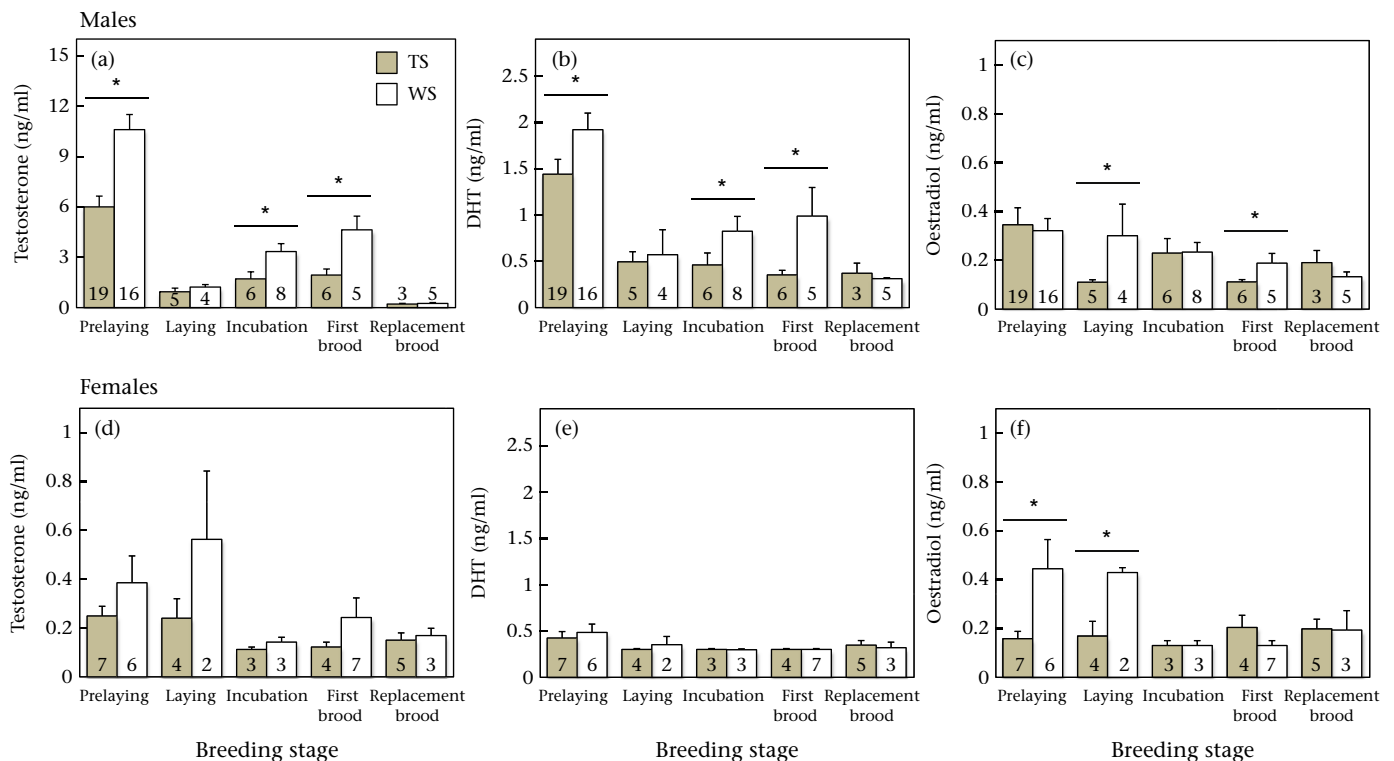


Figure 3. Sex steroid profiles of white-throated sparrow males (a–c) and females (d–f) across the substages of breeding. Note the difference in the scale of the Y axes on the graphs of (a) male and (b) female testosterone. TS: tan-striped morph; WS: white-striped morph. Values are means \pm SE. Numbers in bars denote sample sizes.

Table 3

Results from mixed-model ANCOVA analyses of plasma hormone levels in white-throated sparrows

Dependent	Effect	Males			Females		
		dfs	F	P	dfs	F	P
Plasma T levels	Morph	1, 65	78.3	<0.001	1, 32	4.7	0.038
	Stage	4, 65	62.3	<0.001	4, 32	1.4	0.251
	Morph*stage	4, 65	15.9	<0.001	4, 32	0.8	0.513
	PB time	1, 65	1.6	0.205	1, 32	0.6	0.434
Plasma DHT levels	Morph	1, 65	35.7	<0.001	1, 32	1.5	0.226
	Stage	4, 65	22.2	<0.001	4, 32	0.3	0.855
	Morph*stage	4, 65	5.4	0.004	4, 32	0.5	0.717
	PB time	1, 65	1.9	0.168	1, 32	0.8	0.369
Plasma E2 levels	Morph	1, 65	4.8	0.033	1, 32	5.2	0.030
	Stage	4, 65	2.3	0.068	4, 32	1.2	0.323
	Morph*stage	4, 65	0.7	0.392	4, 32	2.9	0.038
	PB time	1, 65	0.3	0.615	1, 32	0.1	0.870

Models included morph, stage and a morph*stage interaction as fixed main effects, the time from the start of playback to blood sampling (PB time) as a covariate, and year as a random effect. Male and female data were analysed separately. Male models also included individual ID as a random effect to control for repeat sampling; females were not resampled. Significant *P* values are shown in bold.

ability to detect morph-dependent variation in T levels within stages. Female DHT levels, which were low throughout breeding, did not vary according to morph or breeding stage (Table 3, Fig. 3e).

Hormone–Behaviour Relationships

Territorial aggression

In the subset of males ($N = 25$) in which we measured hormone levels within 1 day of a simulated territorial intrusion, WS males sang at higher rates than TS males ($t_{1,24} = 2.3$, $P = 0.04$). Using

multiple regression analysis to parse out the relative influence of morph, breeding stage and steroid levels on singing behaviour, we found that individual variation in the number of songs given in response to the simulated territorial intrusion was explained, in part, by plasma steroids (Fig. 4a–c). In males, the number of songs was positively correlated with plasma levels of T (partial $R^2 = 0.30$, $P < 0.01$; Fig. 4a) and DHT (partial $R^2 = 0.29$, $P < 0.01$; Fig. 4b). Singing also depended on breeding stage in models with T (partial $R^2 = 0.36$, $P = 0.01$) and in models with DHT (partial $R^2 = 0.32$, $P = 0.02$), suggesting that the timing of blood sampling is an important factor in detecting these hormone–behaviour relationships. Indeed, with T or DHT levels and stage in the model, morph no longer predicted variation in the number of songs induced by simulated territorial intrusions (T model: morph partial $R^2 = 0.01$, $P = 0.68$; DHT model: morph partial $R^2 = 0.10$, $P = 0.16$). Thus, androgen levels, together with breeding stage, may explain morph differences in male vocal aggression in this study. Male vocal aggression was not, however, related to E2 levels (partial $R^2 = 0.04$, $P = 0.37$; Fig. 4c).

We collected blood samples after behavioural tests from a limited number of females ($N = 13$). Nevertheless, our results suggest that sex steroids may also be correlated with territorial behaviour in females. For example, the number of songs given by females during simulated territorial intrusion tended to correlate with E2 levels (partial $R^2 = 0.39$, $P = 0.05$; Fig. 4f). There was no evidence, however, that female singing was related to T (partial $R^2 = 0.16$, $P = 0.26$; Fig. 4d) or DHT levels (partial $R^2 = 0.09$, $P = 0.39$; Fig. 4e).

Nestling stage

In the subset of parental males for which we measured hormones after the behavioural tests, WS males sang at higher rates ($t_{1,13} = 2.3$, $N = 14$, $P = 0.04$) but provisioned more ($t_{1,17} = 2.5$,

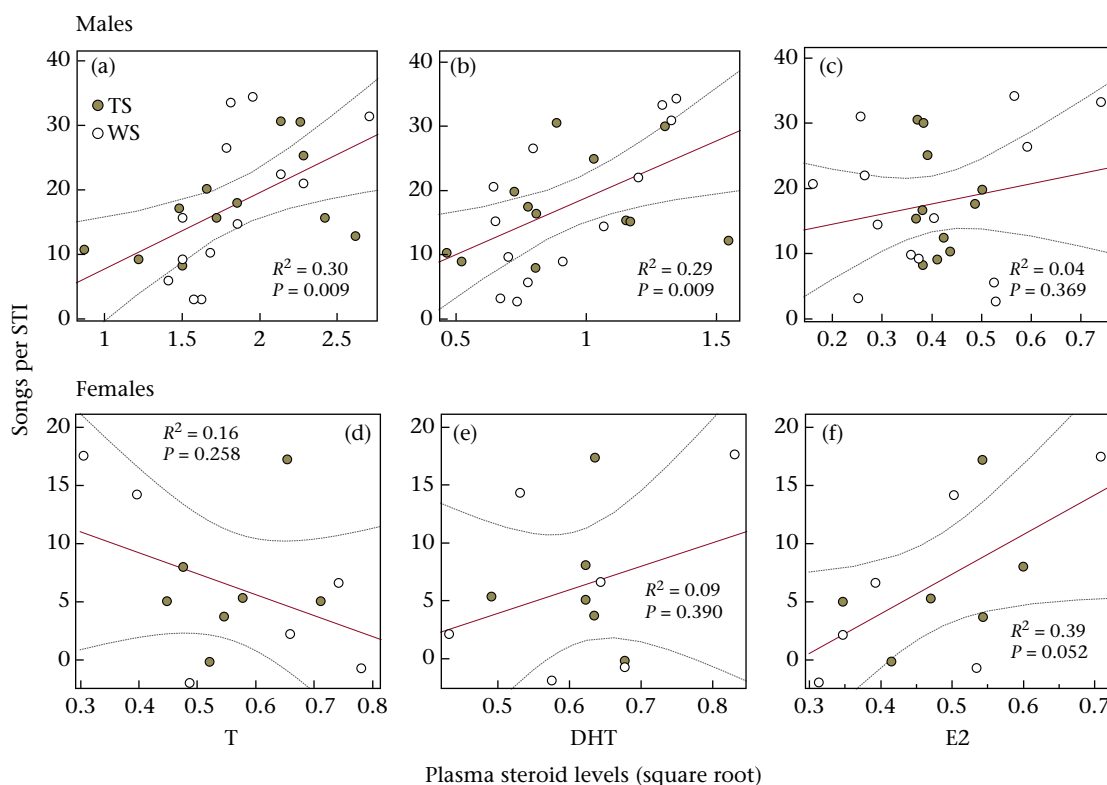


Figure 4. Relationships between plasma steroid levels and number of songs in response to simulated territorial intrusions (STIs) in male (a–c) and female (d–f) white-throated sparrows. Panels are partial regression leverage plots from multiple regression analyses (see text for details). R^2 values are partial regression coefficients; P values are for the effect of hormone level on behaviour. TS: tan-striped morph; WS: white-striped morph.

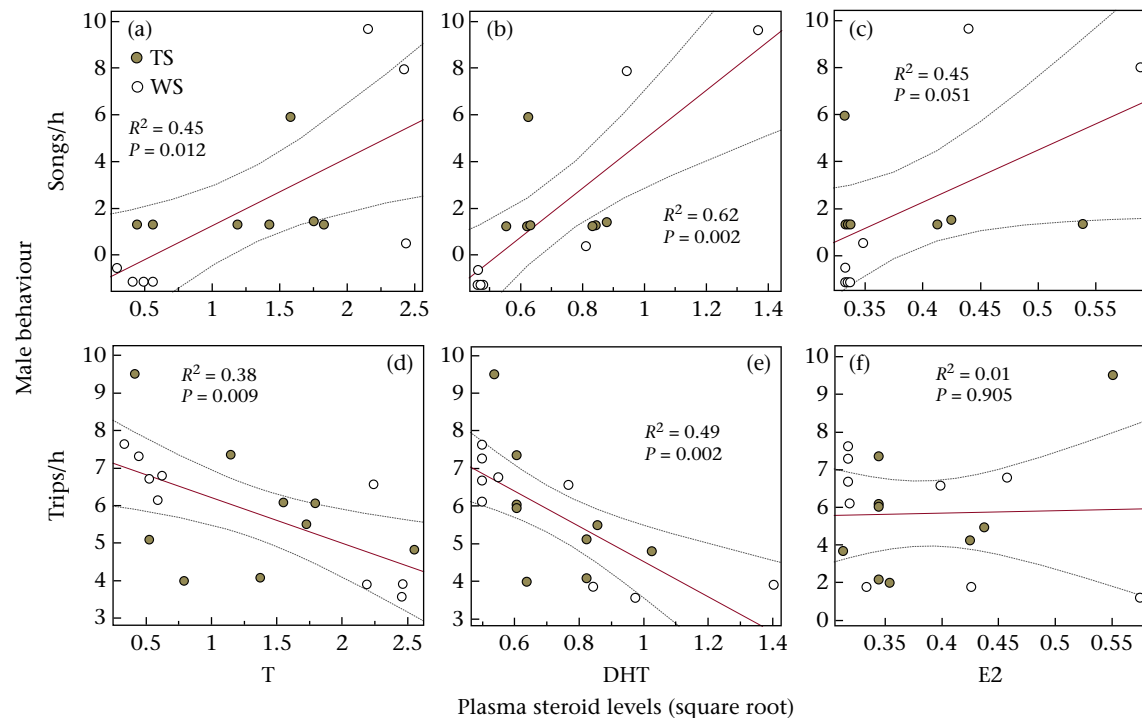


Figure 5. Relationships between sex steroid levels and spontaneous song rates (a–c) and nestling provisioning rates (d–f) in male white-throated sparrows during the nestling stage. Panels are partial regression leverage plots from multiple regression analyses (see text for details). R^2 values are partial regression coefficients; P values are for the effect of hormone level on behaviour. TS: tan-striped morph; WS: white-striped morph.

$N = 18$, $P = 0.03$) than TS males after controlling for nesting attempt. Using multiple regression analysis to parse out the relative influences of morph and sex steroids on singing, we found that male song rates during the nestling stage were positively correlated with plasma T (partial $R^2 = 0.45$, $P = 0.01$; Fig. 5a) and DHT levels ($R^2 = 0.62$, $P < 0.01$; Fig. 5b), and to some extent, E2 levels (partial $R^2 = 0.44$, $P = 0.05$; Fig. 5c). With T, DHT or E2 in the model, the effect of morph on singing was no longer significant, suggesting that sex steroids can explain much of the morphic variation in parental song rates. Note, however, that the partial regression coefficients for morph were not negligible (T model: partial $R^2 = 0.19$, $P = 0.13$; DHT model: partial $R^2 = 0.03$, $P = 0.53$; E2 model: partial $R^2 = 0.34$, $P = 0.16$), which suggests that factors other than sex steroids may also explain morph differences in parental song rates (see also Horton et al., 2014).

For parental behaviour, we again used multiple regression analysis to control for morph effects while exploring the relationship between steroid levels and provisioning. We found that provisioning rates were negatively correlated with plasma T (partial $R^2 = 0.38$, $P < 0.01$; Fig. 5d) and DHT levels (partial $R^2 = 0.49$, $P < 0.01$; Fig. 5e), but were not related to E2 levels (partial $R^2 = 0.01$, $P = 0.91$; Fig. 5f). In contrast to male provisioning rates, female provisioning rates were not related to plasma levels of T (partial $R^2 = 0.0$, $P = 0.95$), DHT (partial $R^2 = 0.01$, $P = 0.74$) or E2 (partial $R^2 = 0.01$, $P = 0.87$).

Cloacal protuberance and testis size

We found that male cloacal protuberance size and testis volume differed between morphs; breeding WS males had larger cloacal protuberances and larger testes than TS males (Table 4). Despite morph differences in male androgen levels males (see above), neither plasma T nor DHT levels were correlated with cloacal protuberance or testis volume in these birds (all $R^2 \leq 0.06$, all $P \geq 0.23$).

DISCUSSION

This study of free-living white-throated sparrows provides new insights into the reproductive behaviour and endocrinology of this unique and promising model species. Collectively, our findings support the hypothesis that behaviour in this species is polymorphic and associated with morph differences in hypothalamic–pituitary–gonadal function (see Maney, 2008). Our results also help to refine our definition of the TS and WS phenotypes by clarifying exactly which behaviours are likely to be polymorphic, and in which sex. Finally, we have shown that morph differences in behaviour in some cases depend critically on breeding stage, which may reflect the influence of extrapair mating opportunity on the degree to which the morphs adopt different strategies.

Territorial Aggression

During territorial intrusions, WS birds of both sexes showed more territorial aggression than TS birds (Tables 1, 2, Fig. 1). WS and

Table 4

Comparison of testis volume and cloacal protuberance between tan-striped (TS) and white-striped (WS) male white-throated sparrows

Volume (mm ³)	TS males	WS males	Morph comparison	
			<i>t</i>	<i>P</i>
Left testis	17.1±0.8	19.1±0.8	2.4	0.025
Right testis	16.5±0.8 (<i>N</i> =22)	20.6±1.2 (<i>N</i> =22)	3.3	0.003
Cloacal protuberance	203±22.5 (<i>N</i> =30)	264±21.6 (<i>N</i> =29)	2.2	0.033

All values are means ± SE in mm³; *t* statistics and *P* values are from an ANCOVA that controlled for Julian day and body mass.

TS females differed in both physical and vocal responses. As has been shown in previous studies (Falls & Kopachena, 2010), WS males sang at much higher rates than TS males. In the context of territorial intrusion, song is an aggressive vocalization (Falls & Kopachena, 2010), and singing by a resident male is the primary deterrent of intrusion in this species (Falls, 1988). Thus, the higher song rates by WS birds in this study supports the idea that this morph displays a more aggressive phenotype than the TS morph. Interestingly, however, morph differences in male aggression were limited to song; physical responses, such as closest approach and number of attacks, were indistinguishable between the morphs (also see Kopachena & Falls, 1993a). This result suggests that, in males, genetic differentiation between the morphs (Davis et al., 2011; Thomas et al., 2008) has affected the physiological mechanisms underlying song but not those underlying other aggressive behaviours.

Territorial aggression in this species depends not only on morph, but also on the degree to which intrusions threaten fitness (Horton et al., 2012). In this study, we demonstrated morph-dependent aggression under the most threatening conditions. We used 'singing' male intruders of each morph at a time when intrusions by males can threaten both usurpation and cuckoldry. Results from previous studies suggest that the breeding stage of the residents, the sex and morph of the intruder, and the sensory modality in which an intrusion is presented (e.g. auditory and/or visual) can all influence the territorial responses of resident birds and, ultimately, whether morph differences in aggression are detected (reviewed by Falls & Kopachena, 2010). For example, resident WS males sang at higher rates than TS males when intrusions were performed before the onset of incubation in the population (Kopachena & Falls, 1993a), but not when they were performed after incubation was well underway (Collins & Houtman, 1999). Moreover, both sexes and morphs show aggression bias; that is, territorial responses depend on both resident and intruder morph (Kopachena & Falls, 1993a; Horton et al., 2012). Whether male or female aggression is the focus of an intrusion experiment, future studies will need to account for intruder morph in their designs, keeping in mind that resident pairs typically consist of one bird of each morph. Overall, it is important to control not only for the morph of the intruder, but also for breeding stage and modality of intrusion in future studies.

Parental Care

In this study, parenting behaviour was clearly related to morph in males but not in females. TS males fed nestlings at higher rates than did WS males, a finding consistent with other studies of parental care in this species (Horton & Holberton, 2010; Knapton & Falls, 1983; Kopachena & Falls, 1993b). Thus, male parental behaviour appears to be reliably polymorphic in this species in that TS males show a more parental phenotype than WS males. In females, however, provisioning effort did not differ between the morphs (Fig. 2d). This finding replicates that of Horton and Holberton (2010), who studied a nearby breeding population in Maine. In contrast, two studies done in Ontario, Canada demonstrated morph differences in provisioning rate for both sexes (Knapton & Falls, 1983; Kopachena & Falls, 1993b). That morph differences in female parental effort are detectable in some cases, but not in others (also see Whillans & Falls, 1990), suggests that environmental factors may interact with morph to affect parental care in females.

Environmental factors change dramatically as the breeding season progresses and affect parenting behaviour accordingly. We found that males altered their behaviour as the nestling stage progressed (Fig. 2a, b). This finding is consistent with other studies demonstrating changes in parental investment across broods or

nesting attempts (Biermann & Robertson, 1981; Robinson et al., 2010). In our study, WS males showed a flexible parental strategy. During first nests, WS males provisioned less but sang more than TS males. WS males then increased their provisioning by 78% for replacement broods, which coincided with a significant decrease in singing (Fig. 2). TS males, on the other hand, provisioned young at relatively high rates but sang at low rates during both nesting attempts. As a result of dramatic changes in parenting and singing in WS males, morph differences in these behaviours were not detectable during replacement nests.

What is the environmental factor that drives such changes? In this species, once breeding progresses beyond first nesting attempts, territorial boundaries stabilize and reneeding becomes sporadic (Falls & Kopachena, 2010); thus, the need for territorial defence diminishes, as do opportunities for extrapair mating. The behavioural adjustments of WS males in this study suggest that they invest in aggression and mating effort at the expense of parental care early in the nestling stage, but as mating opportunities dwindle, they switch to a more parental strategy that resembles that of TS males. Thus, in males, the degree to which the morphs differ in both parenting and singing behaviour may depend directly on the availability of fertile females. In the near-absence of extrapair mating opportunities, we found that TS and WS males invest equally in parenting behaviour.

Related to this idea is the hypothesis that parenting behaviour depends on breeding density and the distribution of the two pair types (Formica et al., 2004; Formica & Tuttle, 2009; Kopachena & Falls, 1993b). This 'social landscape' (Formica & Tuttle, 2009) predicts the intensity of male–male competition and extrapair mating opportunities, and thus may create fitness trade-offs among investments in parental care, territorial aggression and mating effort. The social landscape has previously been invoked to explain why morph differences in parental care were detected in some studies but not in others (Kopachena & Falls, 1993c; Whillans & Falls, 1990). The population we studied here had a lower breeding density and a more equal dispersion of the two pair types than the one studied by Horton and Holberton (2010; B. Horton, personal observation), but we found remarkably similar effects of morph on parental care (Horton & Holberton, 2010). We note that behaviour has been studied in this species at a rather limited number of sites; it will be important to characterize behaviour at more locations in order to understand more fully the relationship between morph and social factors.

Endocrine Correlates of Polymorphic Behaviour

Our study provides new evidence that morph-typic behaviour in this species is related to sex steroid levels. WS males had higher plasma levels of T and DHT than did TS males. The morph difference in T is consistent with previous studies of free-living birds (Spinney et al., 2006; Swett & Breuner, 2009). Our study is the first to describe morph differences in DHT in free-living males. Morph differences in male androgen levels were most apparent in breeding stages during which males differed in territorial aggression (preincubation) or parental behaviour (first broods). By measuring both hormones and behaviour, our study revealed that, in males, androgen levels were directly and positively correlated with challenge-induced singing (Fig. 4a–c), a behaviour that defines morph-typic territorial aggression (Fig. 1; see also Kopachena & Falls, 1993a; Lowther, 1962).

The finding that androgen levels were correlated with morph and behaviour in preparental males is consistent with the stimulatory effects of these hormones on territorial behaviour, including singing, in male birds (Ketterson, Nolan, Cawthorn, Parker, & Ziegenfuss, 1996; Reed et al., 2006). It is thus possible that higher vocal aggression in WS males can be explained by androgen levels. Hormone–behaviour

relationships are reciprocal in nature (Oliveira, 2009; Wingfield, Hegner, Dufty, & Ball, 1990), however, and although exogenous T can promote aggression (Ketterson et al., 1996), aggressive interactions can also increase endogenous T production (Wingfield & Wada, 1989; Wingfield, Ball, Dufty, Hegner, & Ramenofsky, 1987; Wingfield et al., 1990). Because the effects of social interactions on endocrine state may last for days (Wingfield, 2005; Wingfield et al., 1990), and since we measured hormone levels the day after intrusions, the morph differences in T (and DHT) and the correlations between T and singing behaviour reported here may be attributable to the effects of intrusions on endocrine state. It is thus equally plausible that elevated androgens in WS males are the result, not the cause, of morph-typic aggression. Maney et al. (2009) showed that in captive white-throated sparrows, morph differences in singing behaviour persisted even when plasma levels of T were experimentally equalized. Hormone–behaviour relationships in free-living white-throated sparrows must be similarly dissected using hormone manipulations (Horton & Holberton, 2009; Ketterson et al., 1996), which will shed much-needed light on the extent to which hormones contribute to polymorphic behaviour in this species.

Plasma levels of sex steroids also depended on morph in females (Fig. 3). Plasma E2 and T levels were higher in WS females than in TS females. As was the case in males, morph differences in female E2 and T levels were most apparent during stages of peak territorial defence (preincubation). Both androgens and oestrogens facilitate aggressive behaviour in captive white-throated sparrows (Archawaranon & Wiley, 1988). It is thus possible that elevated E2 levels facilitate the relatively high levels of aggression in WS females. It is also the case that hearing male song increases plasma luteinizing hormone (LH) in females of this species (Maney, Goode, Lake, Lange, & O'Brien, 2007), and increases plasma E2 in many songbirds (Bentley, Wingfield, Morton, & Ball, 2000; Morton, Pereyra, & Baptista, 1985), so we cannot rule out the possibility that females had an endocrine response to simulated intrusions that varied according to morph.

We are the first to describe morph differences in sex steroids in free-living females (see Spinney et al., 2006). WS females had higher plasma levels of both T and E2 than did TS females (Fig. 3). Lake et al. (2008) reported that in a captive population, TS females had higher plasma E2 than did WS females, a result opposite to that of the present study. In Lake et al.'s study, the birds were photo-stimulated but not in breeding condition, which probably explains the disparate result. In captivity, WS females do not outrank TS females (Maney, 2008; Watt et al., 1984); thus, if E2 is related to aggressive behaviour, we would not expect WS females to have higher E2 under captive conditions. In the present study, we observed significantly higher song rates, physical aggression and plasma E2 in WS females than in TS females, which is consistent with the hypothesis that morph differences in behaviour and in plasma E2 are functionally related during the breeding season. Alternatively, these behaviours may be more closely related to plasma T, which also depended on morph in females.

Androgen levels were related to morph and behaviour also during the parental stage. We found that in males of both morphs, androgen levels declined from preparental to parental breeding stages (Fig. 3a, b; also see Swett & Breuner, 2009). This pattern of decline is typical of socially monogamous passerines with biparental care (Wingfield et al., 1990). Because androgens are generally antagonistic to parental care in male birds (e.g. Ketterson et al., 1996; Reed et al., 2006), the decline in these hormones is thought to facilitate the transition from nonparental to parental behaviours as breeding progresses (Wingfield et al., 1990). During the nestling stage, we found that male androgen levels were positively correlated with singing but negatively correlated with provisioning (Fig. 5), which is consistent with previously described inverse

effects of these hormones on territorial and parental behaviour in male songbirds (Reed et al., 2006). During first broods, when WS and TS males differed in singing and parenting behaviour, WS males had higher androgen levels. Moreover, increases in parental behaviour and decreases in singing across nesting attempts were mirrored by decreases in plasma androgen levels. Collectively, these findings are consistent with the mounting evidence that androgens mediate territorial and parental behaviour in male songbirds (Hau, 2007; Ketterson & Nolan, 1999; Ketterson et al., 1996; Wingfield et al., 1990). It remains unclear whether the decline in male androgen levels seen in this study, either across breeding stages or nest attempts, is preemptive for meeting the increasing demands of parental care, or responsive to the decreasing demands of territorial defence.

In addition to the endocrine differences described above, we also found that cloacal protuberance and testis volume differed between males of the two morphs; WS males had larger cloacal protuberances and testes than TS males (Table 4). Morph differences in cloacal protuberance size and testis volume were not related to androgen levels in this study; testis volume is also a poor predictor of androgen levels in other *Zonotrichia* spp. (Moore, Perfito, Wada, Sperry, & Wingfield, 2002). In birds, cloacal protuberance and testis size are, however, related to sperm production and storage, respectively, and testis size varies according to mating strategy (reviewed by Birkhead, 1998). Morph differences in these characteristics may thus relate to morph-typic mating strategies (Spinney et al., 2006). Consistent with this hypothesis, free-living WS males, which had larger cloacal protuberances and testes than TS males in this study (also in Spinney et al., 2006; but see DeVoogd et al., 1995; Maney et al., 2009), invest more heavily in extrapair mating, whereas TS males, which had smaller cloacal protuberances and testes, invest more in mate guarding (Tuttle, 2003).

Conclusion

Because white-throated sparrows have a heteromorphic autosomal chromosome that segregates with a life-history strategy, they represent a truly unique opportunity to link genes with social behaviour and to understand the evolution of alternative phenotypes. In this study, we aimed to characterize those phenotypes precisely and provide new insight into which dimensions of behaviour depend on morph and which do not, and under what conditions. First, we found that the morphs did indeed differ in territorial aggression, but this difference was limited to vocal aggression (i.e. singing) in males, whereas physical and vocal aggression differed between the morphs in females. Second, parenting behaviour depended on morph only in males, and only during the first brood of the season. TS and WS males tended to replacement broods at equal rates. Overall, our findings indicate that the degree to which singing and provisioning are polymorphic in males seems to depend on the availability of mating opportunities; as those opportunities decreased, morph differences in behaviour became less robust. We hope that by precisely defining the alternative phenotypes in this species, we will increase its value as a model for understanding the neuroendocrine and genetic bases of vertebrate social behaviour.

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References

- Archawaranon, M., & Wiley, R. H. (1988). Control of aggression and dominance in white-throated sparrows by testosterone and its metabolites. *Hormones and Behavior*, 22, 497–517.
- Ball, G. F., & Balthazard, J. (2008). Individual variation and the endocrine regulation of behavior and physiology in birds: a cellular/molecular perspective. *Philosophical Transactions of the Royal Society B: Biological Sciences*, 363, 1699–1710.
- Bentley, G. E., Wingfield, J. C., Morton, M. L., & Ball, G. F. (2000). Stimulatory effects on the reproductive axis in female songbirds by conspecific and heterospecific male song. *Hormones & Behavior*, 37, 179–189.
- Biermann, G. C., & Robertson, R. J. (1981). An increase in parental investment during the breeding season. *Animal Behaviour*, 29, 487–489.
- Birkhead, T. R. (1998). Sperm competition in birds. *Reviews of Reproduction*, 3, 123–129.
- Birkhead, T. R., & Møller, A. P. (1992). *Sperm competition in birds*. London, U.K.: Academic Press.
- Collins, C. E., & Houtman, A. M. (1999). Tan and white color morphs of white-throated sparrows differ in their non-song vocal responses to territorial intrusion. *Condor*, 101, 842–845.
- Davis, J. K., Mittel, L. B., Lowman, J. J., Thomas, P. J., Maney, D. L., Martin, C. L., et al. (2011). Haplotype-based genomic sequencing of a chromosomal polymorphism in the white-throated sparrow (*Zonotrichia albicollis*). *Journal of Heredity*, 102, 380–390.
- Deviche, P. J., Hurley, L. L., Fokidis, H. B., Lerbour, B., Silverin, B., Silverin, B., et al. (2010). Acute stress rapidly decreases plasma testosterone in a free-ranging male songbird: potential site of action and mechanism. *General and Comparative Endocrinology*, 169, 82–90.
- DeVoogd, T. J., Houtman, A. M., & Falls, J. B. (1995). White-throated sparrow morphs that differ in song production rate differ in the anatomy of some song-related brain areas. *Developmental Neurobiology*, 28, 202–213.
- Falls, J. B. (1988). Does song deter territorial intrusion in white-throated sparrows (*Zonotrichia albicollis*)? *Canadian Journal of Zoology*, 66, 206–211.
- Falls, J. B., & Kopachena, J. G. (2010). White-throated sparrow (*Zonotrichia albicollis*). In A. Poole (Ed.), *The birds of North America online*. No. 128. Ithaca, NY: Cornell Laboratory of Ornithology. Retrieved from <http://bna.birds.cornell.edu/bna/species/128>.
- Formica, V. A., Gonsler, R. A., Ramsay, S., & Tuttle, E. M. (2004). Spatial dynamics of alternative reproductive strategies: the role of neighbors. *Ecology*, 84, 1125–1136.
- Formica, V. A., & Tuttle, E. M. (2009). Examining the social landscapes of alternative reproductive strategies. *Journal of Evolutionary Biology*, 22, 2395–2408.
- Gaunt, A. S., & Oring, L. W. (1999). *Guidelines to the use of wild birds in research* (2nd ed.). Washington, D.C.: The Ornithological Council. Retrieved from <http://www.nmnh.si.edu/BIRDNET>.
- Gross, M. R. (1996). Alternative reproductive strategies and tactics: diversity within sexes. *Trends in Ecology & Evolution*, 11, 92–98.
- Hau, M. (2007). Regulation of male traits by testosterone: implications for the evolution of vertebrate life histories. *BioEssays*, 29, 133–144.
- Horton, B. M., Hauber, M. E., & Maney, D. L. (2012). Morph matters: aggression bias in a polymorphic sparrow. *PLoS One*, 7, e48705.
- Horton, B. M., & Holberton, R. L. (2009). Corticosterone manipulations alter morph-specific nestling provisioning behavior in male white-throated sparrows. *Hormones and Behavior*, 56, 510–518.
- Horton, B. M., & Holberton, R. L. (2010). Morph-specific variation in baseline corticosterone and the adrenocortical response in breeding white-throated sparrows (*Zonotrichia albicollis*). *Auk*, 127, 540–548.
- Horton, B. M., Hu, Y., Martin, C. L., Bunke, B. P., Matthews, E. S., Moore, I. T., et al. (2013). Behavioral characterization of a white-throated sparrow homozygous for the ZAL2^m chromosomal rearrangement. *Behavior Genetics*, 43, 60–70.
- Horton, B. M., Hudson, W. H., Ortlund, E. A., Shirk, S., Thomas, Young, E. R., et al. (2014). Estrogen receptor α polymorphism in a species with alternative behavioral phenotypes. *Proceedings of the National Academy of Sciences of the United States of America*, 111, 1443–1448.
- Jones, J. G. (1987). *Use of space by male white-throated sparrows* (*Zonotrichia albicollis*) (Unpublished doctoral thesis). Toronto, ON: University of Toronto.
- Kempnaers, B., Peters, A., & Foerster, K. (2008). Sources of individual variation in plasma testosterone levels. *Philosophical Transactions of the Royal Society B: Biological Sciences*, 363, 1711–1723.
- Ketterson, E. D., & Nolan, V., Jr. (1999). Adaptation, exaptation, and constraint: a hormonal perspective. *American Naturalist*, 154(Suppl.), S4–S25.
- Ketterson, E. D., Nolan, V., Jr., Cawthorn, M. J., Parker, P. G., & Ziegenfuss, C. (1996). Phenotypic engineering: using hormones to explore the mechanistic and functional bases of phenotypic variation in nature. *Ibis*, 138, 70–86.
- Knapton, R. W., & Falls, J. B. (1983). Differences in parental contribution among pair types in the polymorphic white-throated sparrow. *Canadian Journal of Zoology*, 61, 1288–1292.
- Kopachena, J. G., & Falls, J. B. (1993a). Aggressive performance as a behavioral correlate of plumage polymorphism in the white-throated sparrow (*Zonotrichia albicollis*). *Behaviour*, 124, 249–266.
- Kopachena, J. G., & Falls, J. B. (1993b). Re-evaluation of morph-specific variations in parental behavior of the white-throated sparrow. *Wilson Bulletin*, 105, 48–59.
- Kopachena, J. G., & Falls, J. B. (1993c). Post-fledging parental care in the white-throated sparrow (*Zonotrichia albicollis*). *Canadian Journal of Zoology*, 71, 227–232.
- Lake, J. I., Lange, H. S., O'Brien, S., Sanford, S. E., & Maney, D. L. (2008). Activity of the hypothalamic–pituitary–gonadal axis differs between behavioral phenotypes in female white-throated sparrows (*Zonotrichia albicollis*). *General and Comparative Endocrinology*, 156, 426–433.
- Leys, C., & Schumann, S. (2010). A nonparametric method to analyze interactions: the adjusted rank transform test. *Journal of Experimental Social Psychology*, 46, 684–688.
- Logan, C. A., & Wingfield, J. C. (1995). Hormonal correlates of breeding status, nest construction, and parental care in multiple-brooded northern mockingbirds, *Mimus polyglottos*. *Hormones and Behavior*, 29, 12–30.
- Lowther, J. K. (1961). Polymorphism in the white-throated sparrow, *Zonotrichia albicollis* (Gmelin). *Canadian Journal of Zoology*, 39, 281–292.
- Lowther, J. K. (1962). *Colour and behavioral polymorphism in the white-throated sparrow, Zonotrichia albicollis* (Unpublished doctoral thesis). Toronto, ON: University of Toronto.
- Maney, D. L. (2008). Endocrine and genomic architecture of life history trade-offs in an avian model of social behavior. *General and Comparative Endocrinology*, 157, 275–282.
- Maney, D. L., Erwin, K. L., & Goode, C. T. (2005). Neuroendocrine correlates of behavioral polymorphism in white-throated sparrows. *Hormones and Behavior*, 48, 196–206.
- Maney, D. L., Goode, C. T., Lake, J. I., Lange, H. L., & O'Brien, S. (2007). Rapid neuroendocrine responses to auditory courtship signals. *Endocrinology*, 148, 5614–5623.
- Maney, D. L., Lange, H. S., Raes, M. Q., Reid, A. E., & Sanford, S. E. (2009). Behavioral phenotypes persist after gonadal steroid manipulation in white-throated sparrows. *Hormones and Behavior*, 55, 113–120.
- Mansouri, H., Paige, R. L., & Suries, J. G. (2004). Aligned rank transform techniques for analysis of variance and multiple comparisons. *Communications in Statistics: Theory and Methods*, 33, 2217–2232.
- Moore, I. T., Perfito, N., Wada, H., Sperry, T. S., & Wingfield, J. C. (2002). Latitudinal variation in plasma testosterone levels in birds of the genus *Zonotrichia*. *General and Comparative Endocrinology*, 129, 13–19.
- Morton, M. L., Pereyra, M. E., & Baptista, L. F. (1985). Photoperiodically induced ovarian growth in the white-crowned sparrow (*Zonotrichia leucophrys gambelii*) and its augmentation by song. *Comparative Biochemistry and Physiology A*, 80, 93–97.
- Mulder, R. A., & Cockburn, A. (1993). Sperm competition and the reproductive anatomy of male superb fairy-wrens. *Auk*, 110, 588–593.
- Oliveira, R. F. (2009). Social behavior in context: hormonal modulation of behavioral plasticity and social competence. *Integrative and Comparative Biology*, 49, 423–440.
- Piper, W. H., & Wiley, R. H. (1989). Correlates of dominance in wintering white-throated sparrows: age, sex and location. *Animal Behaviour*, 37, 298–310.
- Piper, W. H., & Wiley, R. H. (1990). The relationship between social dominance, subcutaneous fat, and annual survival in wintering white-throated sparrows (*Zonotrichia albicollis*). *Behavioral Ecology and Sociobiology*, 26, 201–208.
- Reed, W. L., Clark, M. E., Parker, P. G., Raouf, S. A., Arguedas, N., Monk, D. S., et al. (2006). Physiological effects on demography: a long-term experimental study of testosterone's effects on fitness. *American Naturalist*, 167, 667–683.
- Robinson, T. J., Siefferman, L., & Risch, T. S. (2010). Seasonal tradeoffs in reproductive investment in a multi-brooded passerine. *Condor*, 112, 390–398.
- Rosvall, K. A., Bergeon Burns, C. M., Barske, J., Goodson, J. L., Sengelaub, D., Schlenger, B. A., et al. (2012). Neural sensitivity to sex steroids predicts individual differences in aggression: implications for behavioral evolution. *Proceedings of the Royal Society B: Biological Sciences*, 279, 3547–3555.
- Spinney, L. H., Bentley, G. E., & Hau, M. (2006). Endocrine correlates of alternative phenotypes in the white-throated sparrow (*Zonotrichia albicollis*). *Hormones and Behavior*, 50, 762–771.
- Stevenson, T. J., Small, T. W., Ball, G. F., & Moore, I. T. (2012). Variation in the gonadotrophin-releasing hormone-1 and the song control system in the tropical breeding rufous-collared sparrow (*Zonotrichia capensis*) is dependent on sex and reproductive state. *General and Comparative Endocrinology*, 178, 1–7.
- Swett, M. B., & Breuner, C. W. (2008). Interaction of testosterone, corticosterone and corticosterone binding globulin in the white-throated sparrow (*Zonotrichia albicollis*). *Comparative Biochemistry and Physiology A*, 151, 226–231.
- Swett, M. B., & Breuner, C. W. (2009). Plasma testosterone correlates with morph type across breeding substages in male white-throated sparrows. *Physiological and Biochemical Zoology*, 82, 572–579.
- Thomas, J. W., Caceres, M., Lowman, J. J., Morehouse, C. B., Short, M. E., Baldwin, E. L., et al. (2008). The chromosomal polymorphism linked to variation in social behavior in the white-throated sparrow (*Zonotrichia albicollis*) is a complex rearrangement and suppressor of recombination. *Genetics*, 179, 1455–1468.
- Thornycroft, H. B. (1966). Chromosomal polymorphism in the white-throated sparrow, *Zonotrichia albicollis*. *Science*, 154, 1571–1572.
- Thornycroft, H. B. (1975). A cytogenetic study of the white-throated sparrow, *Zonotrichia albicollis*. *Evolution*, 29, 611–621.
- Tuttle, E. M. (1993). *Mate choice and stable polymorphism in the white-throated sparrow* (Unpublished doctoral thesis). Albany, NY: State University of New York.
- Tuttle, E. M. (2003). Alternative reproductive strategies in the white-throated sparrow: behavioral and genetic evidence. *Behavioral Ecology*, 14, 425–432.

- Watt, D. J., Ralph, C. J., & Atkinson, C. T. (1984). The role of plumage polymorphism in dominance relationships of the white-throated sparrow. *Auk*, 101, 110–120.
- Whillans, K. V., & Falls, J. B. (1990). Effects of male removal on parental care of female white-throated sparrows, *Zonotrichia albicollis*. *Animal Behaviour*, 39, 869–878.
- Williams, T. D. (2008). Individual variation in endocrine systems: moving beyond the 'tyranny of the Golden Mean'. *Philosophical Transactions of the Royal Society B: Biological Sciences*, 363, 1687–1698.
- Williams, T. D. (2012). Hormones, life-history, and phenotypic variation: opportunities in evolutionary avian endocrinology. *General and Comparative Endocrinology*, 176, 286–295.
- Wingfield, J. C. (2005). A continuing saga: the role of testosterone in aggression. *Hormones and Behavior*, 48, 253–255.
- Wingfield, J. C., Ball, G. F., Dufty, A. M., Jr., Hegner, R. E., & Ramenofsky, M. (1987). Testosterone and aggression in birds. *American Scientist*, 75, 602–608.
- Wingfield, J. C., & Hahn, T. P. (1994). Testosterone and territorial behaviour in sedentary and migratory sparrows. *Animal Behaviour*, 47, 77–89.
- Wingfield, J. C., Hahn, T. P., Maney, D. L., Schoech, S. J., Wada, M., & Morton, M. (2003). Effects of temperature on photoperiodically induced reproductive development, circulating plasma luteinizing hormone and thyroid hormones, body mass, fat deposition, and molt in mountain white-crowned sparrows, *Zonotrichia leucophrys oriantha*. *General and Comparative Endocrinology*, 131, 143–158.
- Wingfield, J. C., Hegner, R. E., Dufty, A. M., & Ball, G. F. (1990). The challenge hypothesis: theoretical implications for patterns of testosterone secretion, mating systems, and breeding strategies. *American Naturalist*, 136, 829–846.
- Wingfield, J. C., & Wada, M. (1989). Male–male interactions increase both luteinizing hormone and testosterone in the song sparrow, *Melospiza melodia*: specificity, time course and possible neural pathways. *Journal of Comparative Physiology A*, 166, 189–194.